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(54) Title: CALCIUM FREE SUBTILISIN MUTANTS

(57) **Abstract:** Novel calcium free subtilisin mutants are taught, in particular subtilisins which have been mutated to eliminate amino acids 75-83 and which retain enzymatic activity and stability. Recombinant methods for producing same and recombinant DNA encoding for such subtilisin mutants are also provided.

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CALCIUM FREE SUBTILISIN MUTANTS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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5 reasonable terms as provided for by the terms of Grant No. GM42560 awarded by National Institute of Health.

GENERAL OBJECTS OF THE INVENTION

A general object of the invention is to provide subtilisin mutants which have been mutated such that they do not bind calcium.

10 Another object of the invention is to provide DNA sequences which upon expression provide for subtilisin mutants which do not bind calcium.

Another object of the invention is to provide subtilisin mutants which comprise specific combinations of mutations which provide for enhanced thermal stability.

15 Another object of the invention is to provide a method for the synthesis of a subtilisin mutant which does not bind calcium-by the expression of a subtilisin DNA which comprises one or more substitution', deletion or addition mutations in a suitable recombinant host cell.

A more specific object of the invention is to provide class I subtilase
20 mutants, in particular BPN' mutants which have been mutated such that they do not bind calcium.

Another specific object of the invention is to provide DNA sequences which upon expression result in class I subtilase mutants, and in particular BPN' mutants which do not bind calcium.

25 Another specific object of the invention is to provide a method for making subtilisin I-S1 or I-S2 mutants, and in particular BPN' mutants which do not bind

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calcium by expression of a class I subtilase mutant DNA sequence, and more specifically a BPN' DNA coding sequence which comprises one or more substitution, addition or deletion mutations in a suitable recombinant host cell.

Yet another specific object of the invention is to provide mutant subtilisin I-S1 or I-S2, and more specifically BPN' mutants which do not bind calcium and which further comprise particular combinations of mutations which provide for enhanced thermal stability, or which restore cooperativity to the folding reaction.

The subtilisin mutants of the present invention are to be utilized in applications where subtilisins find current usage. Given that these mutants do not bind calcium they should be particularly well suited for use in industrial environments which comprise chelating agents, e.g. detergent compositions, which substantially reduces the activity of wild-type calcium binding subtilisins.

BACKGROUND OF THE INVENTION

(I) Field of the Invention

The present invention relates to subtilisin proteins which have been modified to eliminate calcium binding. More particularly, the present invention relates to novel subtilisin I-S1 and I-S2 subtilisin mutants, specifically BPN' mutants wherein the calcium A-binding loop has been deleted, specifically wherein amino acids 75-83 have been deleted, and which may additionally comprise one or more other mutations, e.g., subtilisin modifications, which provide for enhanced thermal stability and/or mutations which restore cooperativity to the folding reaction.

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(2) Description of the Related Art

Subtilisin is an unusual example of a monomeric protein with a substantial kinetic barrier to folding and unfolding. A well known example thereof, subtilisin BPN' is a 275 amino acid serine protease secreted by *Bacillus amyloliquefaciens*.

5 This enzyme is of considerable industrial importance and has been the subject of numerous protein engineering studies (Siezen et al., Protein Engineering 4:719-737 (1991); Bryan, Pharmaceutical Biotechnology 3(B):147181 (1992); Wells et al., Trends Biochem. Sci. 13:291-297 (1988)). The amino acid sequence for subtilisin BPN' is known in the art and may be found in Vasantha et al., J.
10 Bacteriol. 159:811-819 (1984). The amino acid sequence as found therein is hereby incorporated by reference [SEQUENCE ID NO:1]. Throughout the application, when Applicants refer to the amino acid sequence of subtilisin BPN' or its mutants, they are referring to the amino acid sequence as listed therein.

Subtilisin is a serine protease produced by Gram positive bacteria or by
15 fungi. The amino acid sequences of numerous subtilisins are known. (Siezen et al., Protein Engineering 4:719-737 (1991)). These include five subtilisins from *Bacillus* strains, for example, subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, and mesenticopeptidase. (Vasantha et al., "Gene for alkaline protease and neutral protease from *Bacillus amyloliquefaciens* contain a
20 large open-reading frame between the regions coding for signal sequence and mature protein," J. Bacteriol. 159:811-819 (1984); Jacobs et al., "Cloning sequencing and expression of subtilisin Carlsberg from *Bacillus licheniformis*, Nucleic Acids Res. 13:8913-8926 (1985); Nedkov et al., "Determination of the complete amino acid sequence of subtilisin DY and its comparison with the
25 primary structures of the subtilisin BPN', Carlsberg and amylosacchariticus, Biol. Chem. Hoope-Seyler 366:421-430 (1985); Kurihara et al., "Subtilisin amylosacchariticus," J. Biol. Chem. 247:5619-5631 (1972); and Svendsen et al.,

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"Complete amino acid sequence of alkaline mesentericopeptidase," FEBS Lett. 196:228-232 (1986)).

The amino acid sequences of subtilisins from two fungal proteases are known: proteinase K from *Tritirachium albam* (Jany et al., "Proteinase K from
5 *Tritirachium albam* Limber," Biol. Chem. Hoppe-Seyler 366:485-492 (1985)) and thermomycolase from the thermophilic fungus, *Malbranchea pulchella* (Gaucher et al., "Endopeptidases: Thermomycolin," Methods Enzymol. 45:415-433 (1976)).

These enzymes have been shown to be related to subtilisin BPN', not only through their primary sequences and enzymological properties, but also by
10 comparison of x-ray crystallographic data. (McPhalen et al., "Crystal and molecular structure of the inhibitor eglin from leeches in complex with subtilisin Carlsberg," FEBS Lett. 188:55-58 (1985) and Pahlér et al., "Three-dimensional structure of fungal proteinase K reveals similarity to bacterial subtilisin," EMBO J. 3:1311-1314 (1984)).

15 Subtilisin BPN' is an example of a particular subtilisin gene secreted by *Bacillus amyloliquefaciens*. This gene has been cloned, sequenced and expressed at high levels from its natural promoter sequences in *Bacillus subtilis*. The subtilisin BPN' structure has been highly refined ($R = 0.14$) to 1.3 Å resolution and has revealed structural details for two ion binding sites (Finzel et al., J. Cell.
20 Biochem. Suppl. 10A:272 (1986); Pantoliano et al., Biochemistry 27:8311-8317 (1988); McPhalen et al., Biochemistry 27: 6582-6598 (1988)). One of these (site A) binds Ca^{2+} with high affinity and is located near the N-terminus, while the other (site B) binds calcium and other cations much more weakly and is located about 32 Å away (Figure 1). Structural evidence for two calcium binding sites
25 was also reported by Bode et al., Eur. J. Biochem. 166:673-692 (1987) for the homologous enzyme, subtilisin Carlsberg.

Further in this regard, the primary calcium binding site in all of the subtilisins in groups I-S1 and I-S2 (Siezen et al., 1991, Table 7) are formed from

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almost identical nine residue loops in the identical position of helix C. X-ray structures of the I-S1 subtilisins BPN' and Carlsberg, as well as the I-S2 subtilisin Savinase, have been determined to high resolution. A comparison of these structures demonstrates that all three have almost identical calcium A-sites.

5 The x-ray structure of the class I subtilase, thermitase from *Thermoactinomyces vulgaris*, is also known. Though the overall homology of BPN' to thermitase is much lower than the homology of BPN' to I-S1 and I-S2 subtilisins, thermitase has been shown to have an analogous calcium A-site. In the case of thermitase, the loop is a . ten residue-interruption at the identical site in
10 helix C.

Calcium binding sites are common features of extracellular microbial proteases probably because of their large contribution to both thermodynamic and kinetic stability (Matthews et al., J. Biol. Chem. 249:8030-8044 (1974); Voordouw et al., Biochemistry 15:3716-3724 (1976); Betzel et al., Protein
15 Engineering 3:161-172 (1990); Gros et al., J. Biol. Chem. 266:2953-2961 (1991)). The thermodynamic and kinetic stability of subtilisin is believed to be necessitated by the rigors of the extracellular environment into which subtilisin is secreted, which by virtue of its own presence is protease-filled. Accordingly, high activation barriers to unfolding may be essential to lock the native
20 conformation and prevent transient unfolding and .-proteolysis.

Unfortunately, the major industrial uses of subtilisins are in environments containing high concentrations of metal chelators, which strip calcium from subtilisin and compromise its stability. It would, therefore, be of great practical significance to create a highly stable subtilisin which is independent of calcium.

25 The present inventors have previously used several strategies to increase the stability of subtilisin to thermal denaturation by assuming simple thermodynamic models to approximate the unfolding transition (Pantoliano et al., Biochemistry 26:2077-2082 (1987); Pantoliano et al., Biochemistry 27:8311-8317

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(1988); Pantoliano et al., Biochemistry 28:7205-7213 (1989); Rollence et al., CRC Crit. Rev. Biotechnol. 8:217-224 (1988). However, improved subtilisin mutants which are stable in industrial environments, e.g., which comprise metal chelators, and which do not bind calcium, are currently not available.

5

OBJECTS AND SUMMARY OF THE INVENTION

Accordingly, it is an object of the invention to provide mutated or modified subtilisin enzymes, e.g., class I subtilases, which have been modified to eliminate calcium binding. As used in this invention, the term "mutated or modified subtilisin" is meant to include any serine protease enzyme which has been
10 modified to eliminate calcium binding. This includes, in particular, subtilisin BPN' and serine proteases which are homologous to subtilisin BPN', in particular class I subtilases. However, as used herein, and under the definition of mutated or modified subtilisin enzyme, the mutations of this invention may be introduced into any serine protease which has at least 50%, and preferably 80 %
15 amino acid sequence identity with the sequences referenced above for subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus, mesenticopeptidase, thermitase, or Savinase and, therefore, may be considered homologous.

The mutated subtilisin enzymes of this invention are more stable in the
20 presence of metal chelators and may also comprise enhanced thermal stability in comparison to native or wild-type subtilisin. Thermal stability is a good indicator of the overall robustness of a protein. Proteins of high thermal stability often are stable in the presence of chaotropic agents, detergents, and under other conditions, which normally tend to inactivate proteins. Thermally stable proteins are,
25 therefore, expected to be useful for many industrial and therapeutic applications in which resistance to high temperature, harsh solvent conditions or extended shelf-life is required.

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It has been further discovered that combining individual stabilizing mutations in subtilisin frequently results in approximately additive increases in the free energy of stabilization. Thermodynamic stability has also been shown to be related to resistance to irreversible inactivation at high temperature and high pH.

5 The single-site changes of this invention individually do not exceed a 1.5 Kcal/mol contribution to the free energy of folding. However, these small incremental increases in the free energy of stabilization result in dramatic increases in overall stability when mutations are combined, since the total free energy of folding for most proteins is in the range of 5-15 Kcals/mol (Creighton, Proteins: Structure
10 and Molecular Properties, W.H. Freeman and Company, New York (1984)).

X-ray crystallographic analysis of several combination mutants reveals that conformational changes associated with each mutation tend to be highly localized with minimal distortion of the backbone structure. Thus, very large increases in stability can be achieved with no radical changes in the tertiary protein structure
15 and only minor independent alterations in the amino acid sequence. As previously suggested (Holmes et al, J. Mol. Biol. 160:623 (1982)), contributions to the free energy of stabilization can be gained in different ways, including improved hydrogen bonding and hydrophobic interactions in the folded form and decreased chain entropy of the unfolded enzyme. This is significant since thermostable
20 enzymes generally have more extended half-lives at broader temperature ranges, thereby improving bio-reactor and shelf-life performance.

As noted *supra*, the invention provides subtilisin mutants which comprise one or more deletion, substitution or addition mutations which provide for the elimination of calcium binding. Preferably, this will be effected by deletion,
25 substitution or insertion of amino acids into the calcium A-site, which in the case of class I subtilases comprises 9 amino acid residues in helix C. In the case of subtilisin BPN', the subtilisin mutants will preferably comprise one or more addition, deletion or substitution mutations of the amino acids at positions 75-83,

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and most preferably will comprise the deletion of amino acids 75-83, of SEQUENCE ID NO: 1. The deletion of amino acids 75-83 has been discovered to effectively eliminate calcium binding to the resultant subtilisin mutant while still providing for subtilisin BPN' proteins having enzymatic activity.

5 Such subtilisin mutants lacking amino acids 75-83 of SEQUENCE ID NO: 1 may further include one or more additional amino acid mutations in the sequence, e.g., mutations which provide for reduced proteolysis. It is another object of the invention to produce subtilisin mutants lacking calcium binding activity which have been further mutated to restore cooperativity to the folding
10 reaction and thereby enhance proteolytic stability. It is another object of the invention to provide thermostable subtilisin mutants which further do not bind calcium and comprise specific combinations of mutations which provide for substantially enhanced thermal stability.

 In particular, the subtilisin mutants of the present invention will include
15 subtilisins from Bacillus strains, such as subtilisin BPN', subtilisin Carlsberg, subtilisin DY, subtilisin amylosacchariticus and subtilisin mesenticopeptidase, which comprise one or more deletion, substitution or addition mutations.

 The present invention further provides for subtilisin mutants lacking amino acids 75-83 of SEQUENCE ID NO: 1, which have new protein-protein
20 interactions engineered in the regions around the deletion leading to large improvements in stability. More specifically, mutations at ten specific sites in subtilisin BPN' and its homologues are provided, seven of which individually, and in combination, have been found to increase the stability of the subtilisin protein. Improved calcium-free subtilisins are thus provided by the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. X-ray Crystal structure of S15 subtilisin

A. α -carbon plot shows the positions of mutations as noted. The numbering of wild type subtilisin is kept. Dotted spheres show the position of calcium at the weak ion binding site (B-site) and the former position of the high affinity, binding site (A-site). The A-site loop (dashed line) is absent in this mutant. N- and C- termini are indicated. The N-terminus is disordered (dotted line).

B. Close-up view of the A-site deletion. The loop from S12 subtilisin is shown as a dotted line with the continuous helix of S15. Superimposed is the 3 σ difference electron density (FO12-FO15., phases from S15) showing the deleted A-site loop.

Figure 2. X-ray crystal structure of the calcium A-site region of S12 subtilisin. Calcium is shown as a dotted sphere with one-half the van der Waals radius. Dashed lines are coordination bonds, while dotted lines represent hydrogen bonds under 3.2 Å.

Figure 3. Differential Scanning Calorimetry. The calorimetric scans of apo-S12 ($T_m = 63.5^\circ\text{C}$) and S15 ($T_m = 63.0^\circ\text{C}$) are shown. Measurements were performed with a Hart 7707 DSC (differential scanning calorimetry) heat conduction scanning microcalorimeter as described (Pantoliano et al., Biochemistry 28:7205-7213 (1989)). Sample conditions were 50 mM of glycine, a pH of 9.63, a scan rate of 0.5°C/min. Excess heat capacity is measured in units of $\mu\text{J}/^\circ$. The calorimeter ampoules contained 1.78 mg of protein.

Figure 4. Titration calorimetry of subtilisin S11. The heat of calcium binding for successive additions of calcium are plotted vs. the ratio of $[\text{Ca}]/[\text{P}]$. The data are best fit by a calculated binding curve assuming a binding constant of

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7×10^6 and ΔH equal to 11.3 kcal/mol using equation (1) from the text. For comparison, calculated curves assuming $K_a = 1 \times 10^6$ and 1×10^8 are also shown. In this titration, $[PI] = 100 \mu M$ and the temperature was $25^\circ C$.

Figure 5. Kinetics of calcium dissociation from subtilisin S11 as a function of temperature. $1 \mu M$ subtilisin S11 was added to $10 \mu M$ Quin2 at time = 0. Calcium dissociates from subtilisin and binds to Quin2 until a new equilibrium is achieved. The rate of calcium dissociation is followed by the increase in fluorescence of Quin2 when it binds to calcium.

A. The log of the percent of the protein bound to calcium is plotted vs. time. The kinetics of dissociation at four temperatures are shown. The dissociation follows first order kinetics for the first 25% of the reaction. As this is well before equilibrium is approached, reassociation of calcium can be neglected.

B. Temperature dependence of the rate of calcium dissociation from S15 subtilisin in the presence of excess Quin2, pH 7.4 and over a temperature range of $25-45^\circ C$. The natural log of the equilibrium constant for the transition state (calculated from the Eyring equation) is plotted vs. the reciprocal of the absolute temperature. The line is fit according to equation (3) in the text with $T_0 = 298 K$.

Figure 6. Analysis of subtilisin refolding monitored by circular dichroism (CD).

A. CD spectra are shown for S15 as follows: (1) S15 in 25 mM H_3PO_4 at pH 1.85; (2) S15 denatured at pH 1.85 and then neutralized to pH 7.5 by the addition of NaOH; (3) S15 denatured at pH 1.85 and neutralized to pH 7.5, 30 minutes after the addition of KCl to 0.6 M; and (4) Native S15 subtilisin. Protein concentrations of all samples was $1 \mu M$.

B. Kinetics of refolding of S15. Samples were denatured at pH 1.85 and then the pH was adjusted to 7.5. At time 0, KCl was added to the denatured

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protein. Recovery of native structure was followed at 222 nm at KCl concentrations of 0.3 M and 0.6 M. The 0.6 M sample after 30 minutes of refolding was then used to record the corresponding spectrum in part A.

Figure 7. Kinetics of refolding of S15 as a function of ionic strength.

5 A. The log of the percent unfolded protein is plotted vs. time. The kinetics of refolding are shown at four ionic strengths. The amount of refolding was determined by circular dichroism (CD) from: the increase in negative ellipticity at 222 nm. 100% folding is determined from the signal at 222 nm for native S15 at the same concentration and 0% folding is determined from the signal
10 for acid-denatured S15. The refolding approximately follows first order kinetics for the first 90% of the reaction. Refolding was carried out at 25°C.

 B. The log of first order rate constants for refolding obtained by CD or fluorescence measurements at 25°C were plotted as a function of log of ionic strength. Ionic strength was varied from $I=0.25$ to $I=1.5$. The rate of refolding
15 increases linearly with $\log I$. A ten-fold increase in I results in an approximately 90-fold increase in the refolding rate.

Figure 8. Temperature dependence of the refolding rate of S15 subtilisin in 0.6 M KCl, 23 mM KPO₄, pH 7.3. The natural log of the equilibrium constant for the transition state (calculated from the Eyring equation) is plotted vs.
20 the reciprocal of the absolute temperature. The line is fit according to equation 3 in the text with $T_0=298$ K.

Figure 9. X-ray crystal structure of the weak ion binding region of S15 subtilisin. Coordination bonds are shown as dashed lines. Note the preponderance of charged amino acids.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed *supra*, calcium binding contributes substantially to the thermodynamic and kinetic stability of extracellular microbial proteases. Moreover, with respect to subtilisin, high activation barriers to unfolding may be essential to retain the native conformation and to prevent transient unfolding and proteolysis given the protease-filled environment where subtilisin is secreted and as a result of auto-degradation. The unfolding reaction of subtilisin can be divided into two parts as follows:



where $N(Ca_2)$ is the native form of subtilisin with calcium bound to both sites; $N(Ca)$ is the native form of subtilisin with calcium bound to the high affinity calcium-binding site A (Finzel et al., *J. Cell. Biochem. Suppl.* 10A:272 (1986); Pantoliano et al., *Biochemistry* 27:8311-8317 (1988); McPhalen et al., *Biochemistry* 27:6582-6598 (1988)); N is the folded protein without calcium bound; and U is the unfolded protein. The total free energy of unfolding is therefore equal to $\Delta g_1 + \Delta g_2 + \Delta g_3$. From the binding constant, one can calculate the contribution of calcium to the free energy of subtilisin folding from the following equation:

$$20 \quad \Delta G_{\text{binding}} = -RT \ln (1 + K_a[Ca]).$$

Thus, the contribution of site A to the stability of subtilisin in 10mM calcium is 6.6 kcal/mol at 25°C. The contribution of calcium binding to site B in 10mM calcium and 50mM sodium is only 0.2 kcal/mol. This analysis is at odds with earlier studies which concluded that calcium binding to site B is responsible for the large decrease in the inactivation rate of subtilisin in the presence of millimolar concentrations of calcium (Braxton & Wells, *Biochem.* 31:7796-7801 (1992); Pantoliano et al., *Biochem.* 27: 8311-8317 (1988)).

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Subtilisin is a relatively stable protein whose stability is in large part mediated by the high affinity calcium site (Voordouw et al., Biochemistry 15:3716-3724 (1976); Pantoliano et al., Biochemistry 27:8311-8317 (1988)). The melting temperature of subtilisin at pH 8.0 in the presence of μ molar concentrations of calcium is approximately 75°C and approximately 56°C in the presence of excess EDTA (Takehashi et al., Biochemistry 20:6185-6190 (1981); Bryan et al., Proc. Natl. Acad. Sci. USA, 83:3743-3745 (1986b)). Previous calorimetric studies of the calcium-free (apoenzyme, i.e., protein portion of enzyme) form of subtilisin indicated that it is of marginal stability at 25°C with a ΔG of unfolding of <5 kcal/mol (Pantoliano et al., Biochemistry 28:7205-7213 (1989)). Because calcium is such an integral part of the subtilisin structure, the apoenzyme is thought to be a folding intermediate of subtilisin.

In order to independently examine the two phases of the folding process, the present inventors constructed a series of mutant subtilisins. First, all proteolytic activity was eliminated in order to prevent auto-degradation from occurring during the unfolding and refolding reactions. This may be accomplished, for example, by converting the active-site serine 221 to cysteine.¹ This mutation has little effect on the thermal denaturation temperature of subtilisin, but reduces peptidase activity of subtilisin by a factor of approximately 3×10^4 (Abrahmsen et al., Biochemistry 30:4151-4159 (1991)). This mutant, therefore, allows the folding of subtilisin to be studied without the complications of proteolysis. In the present specification, a shorthand for denoting amino acid substitutions employs the single letter amino acid code of the amino acid to be substituted, followed by the number designating where in the amino acid sequence

¹ The S221A mutant was originally constructed for this purpose. The mature form of this mutant was heterogeneous on its N-terminus, however, presumably due to some incorrect processing of the pro-enzyme.

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the substitution will be made, and followed by the single letter code of the amino acid to be inserted therein. For example, S221C denotes the substitution of serine 221 to cysteine. The subtilisin mutant with this single amino acid substitution is denoted subtilisin S221C. The resulting S221C subtilisin mutant is designated S1.

5 The subtilisin may be further mutated in order to make the relatively unstable apoenzyme easier to produce and purify. Versions of S1 with three or four additional mutations, for example, M50F, Q206I, Y217K and N218S, may also be employed in the method of the present invention. Such further mutations cumulatively increase the free energy of unfolding by 3.0 kcal/mol and increase
10 the thermal denaturation temperature of the apoenzyme by 11.5°C (Pantoliano et al., *Biochemistry* 28:7205-7213 (1989)). The mutant containing the M50F, Q206I, Y217K, N218S and S221C mutations is designated S11 and the mutant containing the M50F, Y217K, N218S and S221C is designated S12.²

15 In order to produce a subtilisin BPN' protein lacking calcium binding activity, the present inventors elected to delete the binding loop in the calcium Asite to engineer a novel calcium-free subtilisin protein. This loop comprises an interruption in the subtilisin BPN' α -helix involving amino acids 63-85 of
SEQUENCE ID NO:1 (McPhalen and James 1988). Residues 75-83 of the subtilisin BPN' protein form a loop which interrupts the last turn of the 14-residue
20 alpha helix involving amino acids 63-85 [SEQUENCE ID NO: 1].³ Four of the

² The specific activities of S11, S12 and S15 against the synthetic substrate, SAAPFna, are the same. (S.A. = 0.0024 U/mg at 25°C, pH 8.0). These measurements were performed on protein freshly purified on a mercury affinity column.

³ This set of nine residues was chosen for deletion, as opposed to 74-82 (those actually belonging to the loop) out of preference for Ala 74 rather than Gly 83 in the resulting continuous helix. Alanine has a higher statistical likelihood for occurrence in α -helix, due to glycine's broader
(continued...)

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carbonyl oxygen ligands to the calcium are provided by a loop composed of amino acids 75-83 [SEQUENCE ID NO: 1]. The geometry of the ligands is that of a pentagonal bipyramid whose axis runs through the carbonyls of amino acids 75 and 79. On one side of the loop is the bidentate carboxylate (D41), while on the other side is the N-terminus of the protein and the side chain of Q2. The seven coordination distances range from 2.3 to 2.6 Å, the shortest being to the aspartyl carboxylate. Three hydrogen bonds link the N-terminal segment to loop residues 78-82 in parallel-beta arrangement. A high affinity calcium binding site is a common feature of subtilisins which make large contributions to their high stability. By binding at specific sites in the subtilisin tertiary structure, tertiary structure, calcium contributes its binding free energy to the stability of the native state. In the present invention, site-directed mutagenesis was used to delete amino acids 75-83 of SEQUENCE ID NO: 1, which creates an uninterrupted helix and abolishes the calcium binding potential at site A (Figure 1A and 1B).

The present inventors believed that a stabilization strategy based around calcium binding would allow survival in the extracellular environment. Since the major industrial uses of subtilisins are in environments containing high concentrations of metal chelators, it was of great practical significance for the present inventors to produce a stable subtilisin which is independent of calcium and, therefore, unaffected by the presence of metal chelating agents. Thus, stabilizing mutations in subtilisin can be classified into three groups: 1) stabilizing only in calcium, 2) stabilizing only in chelants; 3) stabilizing in both conditions (Table 1). From this partitioning it is evident that the mechanism of thermal inactivation differs depending on whether the calcium sites are occupied. To understand why this is so, one must understand how the kinetics of inactivation

³(...continued)

range of accessible backbone conformations.

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are related to the kinetics of unfolding and how the kinetics of unfolding are related to the kinetics of calcium loss.

While the present inventors chose to eliminate calcium binding by the removal of these amino acids (*i.e.* amino acids at positions 75-83), it should be possible to eliminate calcium binding by other mutations, *e.g.*, substitution of one or more of the amino acids at positions 75-83 with alternative amino acids and by insertion, substitution and/or deletion of amino acids proximate to positions 75-83. This may also be accomplished by site-specific mutagenesis.

Additionally, because this loop is a common feature of subtilisins, it is expected that equivalent mutations for other subtilisins, in particular class I subtilases, *e.g.*, by site-specific mutagenesis, will likewise eliminate calcium binding and provide for enzymatically active mutants.

In particular, the present inventors synthesized by site-specific mutagenesis three subtilisin BPN' DNA's which have been mutated to eliminate amino acids 75-83 involved in calcium binding and which further comprise additional substitution mutations. These mutated subtilisin BPN' DNA's, upon expression of the DNA, provide for subtilisin proteins having enhanced thermal stability and/or which are resistant to proteolysis.

The specific subtilisin BPN' mutants synthesized by the present inventors are designated in this application as S15, S39, S46, S47, S68, S73, S79, S86, S88 and pS149. The specific point mutations set forth in the present application identify the particular amino acids in the subtilisin BPN' amino acid sequence, as set forth in SEQUENCE ID NO: 1, that are mutated in accordance with the present invention. For example, the S15 mutant comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: S221C, N218S, M50F and Y217K. The S39 mutant similarly comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: S221C, P5A, N218S, M50F and Y217K. The S46 mutant

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comprises a deletion of amino acids 75-83 and additionally, comprises the following substitution mutations: M50F, Y217K and N218S. The S47 mutant similarly comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: P5A, N218S, M50F and Y217K. The S68 mutant comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: P5S, N218S, M50F and Y217K. The S73 mutant comprises a deletion of amino acids 75-83 as well as the following substitution mutations: Q2K, M50F, A73L, Q206V, Y217K and N218S. The S79 mutant comprises a deletion of amino acids 75-83 and additionally comprises the following substitution mutations: Q2K, M50F, A73L, Q206C, Y217K and N218S. The S86 mutant comprises a deletion of amino acids 75-83 as well as the following substitution mutations: Q2K, S3C, M50F, A73L, Q206C, Y217K and N218S. The S88 mutant comprises a deletion of amino acids 75-83 as well as the following substitution mutations: Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, and Q271E. Finally, the pS149 mutant comprises a deletion of amino acids 75-83 as well as the mutations present in the S88 mutant and the following substitution mutations: S9A, I131L, E156S, G166S, G169A, S188P, N212G, K217L and T254A. The specific activities of the proteolytically active S46, S47, S68, S73, S79, S86, S88 and pS149 subtilisins have been found to be similar or enhanced in relation to the wild-type enzyme.

Applicants also consider as part of their invention Δ 75-83 subtilisin mutants which contain one or more of the following mutations: Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, Q271E, S9A, I131L, E156S, G166S, G169A, S188P, N212G, K217L and T254A. Furthermore, applicants consider as part of their invention Δ 75-83 subtilisin mutants which contain one or more substitutions selected from the group consisting of S9A, I131L, E156S, G166S, G169A, S188P, N212G, K217L and T254A, and optionally together with

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at least one or more substitutions selected from the group consisting of Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, and Q271E.

5 The various $\Delta 75-83$ subtilisins which were synthesized by the inventors are shown in Tables 1 and 2, below. The particular points of mutation in the amino acid sequence of subtilisin BPN' amino acid sequence, as set forth in SEQUENCE ID NO:1, are identified. The synthesis of these mutants is described in more detail *infra*.

TABLE I: Subtilisin Mutations

	S221C	P5A	Δ75-83	N218S	M50P	Q206I	Y217K	Q271E	Q3K	A73L	K43N	Q206C	S3C
BPN*	-	-	-	-	-	-	-	-	-	-	-	-	-
S1*	+	-	-	-	-	-	-	-	-	-	-	-	-
S11*	+	-	-	+	+	+	+	-	-	-	-	-	-
S12*	+	-	-	+	+	-	+	-	-	-	-	-	-
S13*	+	-	+	+	+	-	+	-	-	-	-	-	-
S39	+	+	+	+	+	-	+	-	-	-	-	-	-
S46	-	-	+	+	+	-	+	-	-	-	-	-	-
S47	-	+	+	+	+	-	+	-	-	-	-	-	-
S68	-	P5S	+	+	+	-	+	-	-	-	-	-	-
S73	-	-	+	+	+	-	+	-	-	-	-	-	-
S79	-	-	+	+	+	-	+	+	+	+	-	+	-
S86	-	-	+	+	+	-	+	+	+	+	-	+	+
S88	-	P5S	+	+	+	-	+	+	+	+	+	+	+

The plus signs show that a subtilisin contains a particular mutation. X-ray crystal structure of wild type, S12 and S15 have been determined to 1.8Å.

*S1, S11, S12, S15 and S39 are low activity mutants constructed to aid in the evaluation of structure and conformational stability.

TABLE 1: Subtilisin Mutations (cont.)										
	S88	S9A	I31L	E156S	G166S	G169A	N212G	S188P	K217L	T254A
pS149*	S8									254

*pS149 contains all of the mutations present in S88, in addition to the mutations depicted in the table.

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In order to understand the contribution of calcium binding to the stability of subtilisin, the thermodynamics and kinetics of calcium binding to the high affinity calcium A-site were measured by microcalorimetry and fluorescence spectroscopy. Calcium binding is an enthalpically driven process with an
5 association constant (K_a) equal to $7 \times 10^6 \text{ M}^{-1}$. The kinetic barrier to calcium removal from the A-site (23 kcal/mol) is substantially larger than the standard free energy of binding (9.3 kcal/mol). The kinetics of calcium dissociation from subtilisin (e.g. in excess EDTA) are accordingly very slow. For example, the half-life ($t_{1/2}$) of calcium dissociation from subtilisin, i.e., the time for half of the
10 calcium to dissociate from subtilisin, is 1.3 hours at 25°C.

X-ray crystallography shows that except for the region of the deleted calcium-binding loop, the structure of the subtilisin mutants and the wild type protein are very similar. The N-terminus of the wild-type protein lies beside the site A loop and furnishes one calcium coordination ligand, the side chain oxygen
15 of Q2. In $\Delta 5-83$ subtilisin, the loop is -one, leaving residues 1-4 disordered. These first four residues are disordered in the X-ray structure since all its interactions were with the calcium loop. N-terminal sequencing confirms the first four amino acids are present, confirming that processing occurs at the normal site. The helix is shown to be uninterrupted and shows normal helical geometry over its
20 entire length. X-ray crystallography further shows that the structures of subtilisin with and without the deletion superimpose with a root mean square (r. m. s.) difference between 261 α -carbon positions of 0.17 Å and are remarkably similar considering the size of the deletion. Diffuse difference density and higher temperature factors, however, indicate some disorder in the newly exposed
25 residues adjacent to the deletion.

While the elimination of calcium binding is advantageous since it produces proteins that are more stable in the presence of metal chelators, it is disadvantageous in at least one respect. Specifically, the elimination of the

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calcium loop without any other compensating mutations results in the destabilization of the native state relative to the partially folded states and, therefore, a loss of cooperativity in folding. The present inventors thus sought to further genetically engineer the subtilisin S15 BPN' protein lacking amino acids
5 75-83 in order to restore cooperativity to the folding reaction. In most well designed proteins all parts of the molecule are interdependent, making the unfolding reaction highly cooperative. Cooperativity of the folding reaction allows proteins to achieve sufficient stabilities of the native state for proper function since the overall stability of the native conformation is roughly the sum of
10 all local interactions.

Therefore, while the $\Delta 75-83$ subtilisin is an example of an engineered subtilisin which is active and stable in the absence of calcium, the present inventors sought to improve this protein by further mutation. The design of a particular highly stable calcium-free subtilisin relies on an iterative engineering
15 cycle. The present inventors found that the requisite first step in the cycle was to greatly diminish the proteolytic activity of subtilisin. This is necessary because calcium contributes greatly to the conformational stability of subtilisin and the early versions of calcium-free subtilisin are susceptible to proteolysis. After reducing the susceptibility to proteolysis, the next step in the cycle was to
20 eliminate sequences essential for calcium binding, i.e., the A-site. Although the S15 $\Delta 75-83$ subtilisin is much less stable than the wild type subtilisin in the presence of calcium, this mutant is more stable than wild type subtilisin in the presence of the metal chelator EDTA.

Accordingly, the third step was to improve the stability of the calciumfree
25 subtilisin protein. To improve the stability of calcium-free subtilisin, the present inventors next tried to create a home for the disordered N-terminal residues. In order to create a highly stable calcium-free subtilisin, the N-terminal part of the protein which is destabilized by the deletion of the calcium A-loop may be

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modified. For example, the N-terminus which is disordered may be deleted or extended. This, however, is problematic because the requirements for processing the propeptide from the mature protein are not known. It is known, however, that the processing site is not determined by amino acid sequence since mutants Y1A (the C-terminus of the propeptide), A1C and Q2R do not alter the site of cleavage. It is also known that the native structure of the N-terminus in subtilisin does not determine the cleavage site because the $\Delta 75-83$ variants are processed correctly. Since it is not yet known how to alter the processing site, interactions with the existing N-terminus may be optimized.

Examination of the structure of S15 subtilisin revealed numerous possibilities for improving stability of the mutant enzyme. The regions of the structure most affected by the deletion are the N-terminal amino acids 1-8, the 36-45 ω -loop, the 70-74 α -helix the 84-89 helix turn and the 202-219 β -ribbon. As previously stated, the first four residues in $\Delta 75-83$ subtilisin are disordered in the x-ray structure since all its interactions had been with the calcium loop. N-terminal sequencing shows, however, that the first four amino acids are present confirming that processing occurs at the normal site. Other than the N-terminus, there are three other residues whose side chain conformations are distinctly different from wild type. Y6 swings out of a surface niche into a more solvent-exposed position, as an indirect effect of the destabilization of the N-terminus. D41, a former calcium ligand, and Y214 undergo a coordinated rearrangement, forming a new hydrogen bond. The B-factors of all three residues increase significantly due to the deletion of amino acids 75-83. In addition, S87 and A88 do not change conformation but exhibit significantly increased B-factors. P86 terminates the α -hela from which the calcium loop was deleted. In view of the above, other mutations at one or more of the above mentioned sites, or at the amino acids proximate thereto, will provide for subtilisin BPN' mutants comprising greater enzymatic activity or increased stability.

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There are several logical strategies for remodeling this region of the protein to produce subtilisin BPN' mutants comprising greater enzymatic activity or increased stability. Since the N-terminal four amino acids are disordered in the x-ray structure, one possible approach would be to delete them from the protein.

5 The requirements for processing the propeptide from the mature protein are not understood, however. Inserting or deleting amino acids from the N-terminal region is, therefore, problematic. For this reason insertions and deletions in the N-terminal region were avoided in favor of amino acid substitutions. Many of the original amino acids in the above described regions of subtilisin which
10 interacted with the amino acids 75-83 loop can be assumed to no longer be optimal. It was, therefore, possible to increase the stability of the molecule by substituting, deleting or adding at least one amino acid at positions whose environment was changed by the 75-83 deletion.

The first attempt was to mutate the proline at position 5 to alanine to create
15 more flexibility at position 5. This increased flexibility allows the N-terminus to try to find a unique position along the new surface of the protein, created by deletion of the calcium loop. Once the N-terminus assumes a unique location its local interactions may then be optimized.

The P5A mutation was made to try to create more flexibility for the N-
20 terminus and allow it to find a unique position along the new surface of the protein that was created by deletion of the calcium loop. In the native structure, the first five amino acids are in an extended conformation and form β -pair hydrogen bonds with the calcium loop as well as the Q2 side chain interaction with the calcium. The proline at position 5, which is conserved among seven bacterial subtilisins
25 which have a homologous calcium A-site, may help stabilize the extended conformation. The P5A mutation in $\Delta 75-83$ subtilisin should thus result in an increase in the cooperativity of the unfolding reaction. The X-ray structure of this variant has been determined to 1.8 Å.

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In *toto*, the present inventors selected amino acids at ten different positions whose environment had changed substantially for substitution. A mutagenesis and screening procedure was developed in order to screen all possible substitutions at a particular site. The technique for generating and screening subtilisin variants
5 involves *in vitro* mutagenesis of the cloned subtilisin gene, expression of the mutated genes in *B. subtilis*, and screening for enhanced stability.

For example, site-directed mutagenesis was performed on the S46 subtilisin gene using oligonucleotides which were degenerate at one codon. The degenerate codon contained all combinations of the sequence NNB, where N is any of the
10 four nucleotides and B is T, C or G. The 48 codons represented in this population encode for all twenty amino acids but exclude the ochre and amber termination codons. The mutagenized genes were used to transform *B. subtilis*. Examples of particular mutations are shown in Table II as follows:

Table II - Site-directed mutagenesis

Region of protein	Site	Stabilizing mutations	Mutagenic Oligonucleotide
N-terminus:	Q2	K, W, L	AC GCG TAC GCG NNB TCC GTG CCT TAC
	S3	C*	GCG TAC GCG AAG MMB GTG CCT TAC CG
	V4	none	C GCG AAG TCC NNB OCT TAC GGC G
	P5	S	CAG TCC GTG NNB TAC GGC GTA TC
36-44 omega loop:	D41	A	GAT TCT TCT CAT CCT NNB TTA AAG GTA GC
	K43	R,N	CAT CCT GAT TTA NNB GTA GCA GGC GG
63-85 α -helix:	A73	L,Q	GGC AVA GTT NNB GCT GTT CCG
	A74	none	C ACA GTT GCG NNB GTT GCG CCA AG
202-220 β -ribbon:	Q206	L,V,W,C*	C GTA TCT ATC MMB AGC ACG CTT CC
	Y214	none	CCT CGA AAC AAA NTN GGG GCG AAA TC

*Double cysteine mutations at positions 3 and 206 have been found to be as stabilizing as a disulfide bond.

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To have a 98 % chance of finding tryptophan, glutamine, glutamate or methionine in the mutant population, one must screen about 200 mutant clones. Each of those codons is represented by only one of the 48 codons contained in the population of sequences NNB. Codons for all other amino acids are represented
5 by at least two codons in the population and would require screening of about 1 00 mutant clones to have a 98 % chance of being represented in the mutant population.

To identify the optimum amino acid at a position, mutants were screened for retention of enzymatic activity at high temperature. 100 μ l of media was
10 dispensed in each of the 96 wells of a microliter dish. Each well was inoculated with a *Bacillus* transformant and incubated at 37' with shaking. After 18 hours of growth, 20 μ l of culture was diluted into 80 μ l of 100 mM Tris-HCl, pH 8.0 in a second microliter dish. This dish was then incubated for one hour at 65'C. The dish was allowed to cool to room temperature following the high temperature
15 incubation and 100 μ l of 1 mM SAAPF-pNA was added to each well. The wells which cleaved the pNA (turned yellow) quickest were deemed to contain the most heat resistant subtilisin mutant. Once preliminary identification of a stable mutant was made from the second microliter dish, the *Bacillus* clone in the corresponding well in the first microliter dish was grown up for further analysis.

20 The screening procedure identified stabilizing mutations at seven of the ten positions which were examined. As noted, these amino acid positions were selected at positions of the protein whose environment has changed substantially by virtue of the calcium domain deletion. No mutations were identified at positions 4, 74 and 214 which by themselves significantly increased the half-life of
25 the mutant relative to the parent subtilisin. However, at position 214 the effect of only hydrophobic amino acids was screened. No mutations were found at positions 5, 41 and 43 which resulted in measurable but modest increases in stability. Moreover, several mutations were found at positions at 2, 3, 73, and

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206 which significantly increased the half-life of the mutant relative to the parent subtilisin. These stabilizing mutations are shown in Table III as follows:

Table III - Stabilizing Mutations

	Region of protein	Site	Increase
5	N-terminus:	Q2K	2.0-fold
	63-85 α -helix:	A73L	2.6-fold
	202-220 β -ribbon	Q206V	4.5-fold
	N-terminus - β -ribbon	S3C-Q206C (disulfide)	14-fold

Stabilizing amino acid modifications at positions 2(K), 73(L) and 206(V)
10 were then combined to create subtilisin S73. The properties of S73 subtilisin as well as S46, S79 and S86 are summarized in Table IV.

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Table IV

	Mutant	Mutations ¹	Specific activity ²	Half-life (60 °C) ³	Increase
	S46	-	100U/mg	2.3 min	
5	S73	Q2K A73L Q206V	160U/mg	23 min	11-fold
10	S79	Q2K A73L Q206C	N.D. ⁴	18 min	8-fold
	S86	Q2K, S3C ⁵ A73L Q206C ⁵	85U/mg	80 min	35-fold
15	1)	All of subtilisins S46, S73, S79 and S86 contain the mutations M50F, Y217K and N218S and Q271E.			
	2)	Specific activity is measured against succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide(SAAPF-pNA) in 10mM Tris-HCl, pH 8.0 at 25°C.			
20	3)	Half-life is measured at 60°C in 10mM Tris-HCl, pH 8.0 50mM NaCl and 10mM EDTA.			
	4)	Not determined.			
	5)	Disulfide bond was formed between the cysteines at positions 3 and 206. The formation of a disulfide bond was confirmed by measuring the radius of gyration of the denatured protein by gel electrophoresis.			

25 In many cases, the choice of amino acid at a particular position will be influenced by the amino acids at neighboring positions. Therefore, in order to find the best combinations of stabilizing amino acids, it will be necessary in some cases to vary the amino acids at two or more positions simultaneously. In particular, this was effected at positions 3 and 206 with amino acids whose side

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chains can potentially interact. It was determined that the best combination of modifications was cysteine modifications at positions 3 and 206. This modification was denoted as S86. Because of the close proximity and suitable geometry between the cysteines at these two positions a disulfide cross-link forms spontaneously between these two residues.

The stability of the S86 subtilisin was studied in relation to S73. It was found that the half-life of S86 is 80 minutes at 60°C in 10mM Tris-HCl, pH 8.0, 50mM NaCl and 10mM EDTA, a 3.2-fold enhancement relative to S73 subtilisin. By contrast, a 3-206 disulfide cross-link would not be able to form in native subtilisins which contain the calcium A-site because the 75-83 binding loop separates the N-terminal amino acids from the 202-219 B ribbon. Therefore, the enhancement in stability which occurs in the subject S86 mutant lacking the 75-83 binding loop will likely not be observed with native subtilisins similarly cysteine modified at these positions.

It is expected that similar enhancement in stability will be inherent to other subtilisins of the I-S1 and I-S2 group if their calcium loops were deleted (see Siezen et al, Protein Engineering, 4, pp. 719-737 at Figure 7). This is a reasonable expectation based on the fact that the primary calcium site in these different subtilisins are formed from almost identical 9 residue loops comprised in the identical position of helix C.

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X-ray structures of the I-SI subtilisins BPN and Carlsberg, as well as the I-S2 subtilisin (savinase), have been determined to high resolution. Comparison of these structures demonstrates that all three have almost identical calcium A-sites.

The x-ray structure of the class I subtilase, thermitase from

5 *Thermoactinomyces vulgaris*, is also known. Though the overall homology of BPN' to thermitase is much lower than the homology of BPN' to I-SI and I-S2 subtilisins, thermitase has been shown to have an analogous calcium A-site. In the case of thermitase, the loop is a ten residue interruption at the identical site in helix C.

10 Thus, it is expected that the stabilizing mutations exemplified herein will impart similar beneficial effects on stability for the calcium loop-deleted versions of other class I subtilases.

 The stability of S73, S76 and S86 subtilisins relative to S46 subtilisin was compared by measuring their resistance to thermal inactivation at 60°C in 10mM
15 Tris-HCl, pH 8.0, 50mM NaCl and 10mM EDTA. Aliquots were removed at intervals and the activity remaining in each aliquot was determined. Under these conditions, the half-life of S46 subtilisin is 2.3 minutes and the half-life of S73 is 25 minutes (Table IV).

 In order to identify other mutants having increased stability any
20 mutagenesis technique known by those skilled in the art may be used. One example of such a technique for generating and screening subtilisin variants

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involves three steps: 1) *in vitro* mutagenesis of the cloned subtilisin gene; 2) expression of mutated genes in *B. subtilis*, and 3) screening for enhanced stability. The key element in the random mutagenesis approach is being able to screen large numbers of variants.

5 Although random mutagenesis may be employed, the mutagenesis procedure described above allows for mutations to be directed to localized regions of the protein (e.g., the N-terminal region). As noted supra, the S46, S47, S68, S73, S79 and S86 mutants (which comprise the active-site S221) were found to be enzymatically active. It is expected that other substitutions may be identified
10 which provide for equivalent or even greater stability and activity.

 The activities of examples of the calcium-free subtilisin mutants of the present invention against the substrate sAAPF-pNA in Tris-HCl, pH 8.0 and 25°C are given in Table V as follows:

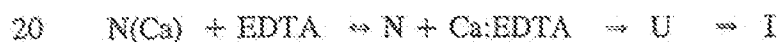
-33-

Table V

	Subtilisin	Specific activity	Half-life (55°C)
	BPN'	80 U/mg	2 min
	S12	0.0025 U/mg	N.D. ¹
5	S15	0.0025 U/mg	N.D. ¹
	S39	0.0025 U/mg	N.D. ¹
	S46	125 U/mg	22 min
	S47	90 U/mg	4.7 min
	S68	~ 100 U/mg	25 min
10	¹ Half-lives were not determined for inactive subtilisins.		

As shown above, the subtilisin mutants S46, S47, S68, S73, S79 and S86 have enhanced catalytic activity in comparison with subtilisin BPN'. Changes in catalytic efficiency due to the deletion were not expected because of the fact that the active site of subtilisin is spatially distant from the calcium A-site.

15 The stability of these mutant subtilisins was compared to native subtilisin BPN' by measuring their resistance to thermal inactivation. Since the stability of the calcium-free subtilisin mutants should be unaffected by metal chelating agents, the experiment was carried out in EDTA. Thermal inactivation in EDTA is a two step process as shown in the following mechanism:



The rate of calcium dissociation with the rate of unfolding as a function of temperature for an inactive variant of subtilisin BPN' was compared in Bryan et al

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(*Biochem. 31:4937-4945* (1992)) . Repartitioning of calcium from site A into a strong chelator occurs at a rate 5 hour^{-1} at 45°C . The kinetic barrier to calcium removal is 23 kcal/mol. Calcium is an integral part of the subtilisin structure and its association or dissociation requires significant but transient disruption in surrounding protein-protein interactions. This disruption in structure would explain the high activation energy and slow kinetics of calcium binding and dissociation. For example, breaking main-chain hydrogen bonds between the N-terminal region and the 75-83 loop region would allow the relatively buried calcium a passageway into or out of the protein. Global unfolding in 10mM EDTA at 45°C is much slower than calcium dissociation, however, occurring at a rate of 0.04 hour^{-1} , with an activation energy of $\sim 60 \text{ kcal/mol}$. Thus the predominant mechanism of inactivation in EDTA is calcium dissociation followed by unfolding and loss of activity.

Because calcium binding reaches equilibrium quickly relative to the rate of unfolding, mutations which stabilize in EDTA must stabilize apo-subtilisin. Increasing the binding constant for one of the calcium sites would not help unless the increase in binding affinity were enormous. Consider a typical experiment in which 1mM EDTA is added to $100 \mu\text{g/ml}$ subtilisin ($3.6 \mu\text{M}$) bound to a stoichiometric amount of calcium. The calcium will partition between subtilisin and EDTA according to the equation:

$$[\text{SCa}]/[\text{S}_{\text{total}}] = K_{\text{S-Ca}} [\text{S}]/(1 + K_{\text{S-Ca}} [\text{S}] + K_{\text{E-Ca}} [\text{E}])$$

-35-

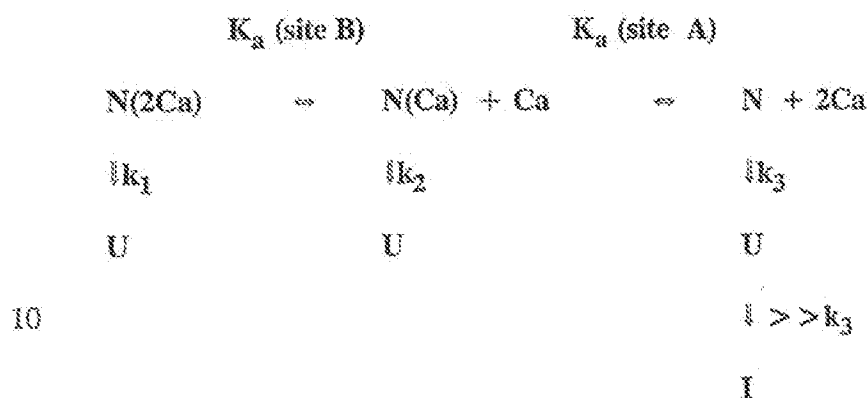
where $[SCa]/[S_{total}]$ is the fraction of subtilisin bound to calcium, $[S] \sim$ total subtilisin and $[E] \sim$ total EDTA. Since the binding constant of subtilisin for calcium at site A (K_{S-Ca}) = $7 \times 10^6 M^{-1}$ and the binding constant of EDTA for calcium (K_{E-Ca}) = $2 \times 10^8 M^{-1}$, then less than 0.02% subtilisin would be bound to calcium at equilibrium. Examples of mutations which stabilize apo-subtilisin are M50F and the disulfides C22- C87 and C206-C216 (Pantoliano et al, *Biochem.* 28:7205-7213 (1989)). The irony is that a mutation which preferentially stabilizes apo-subtilisin relative to the bound form, will weaken calcium binding and catalyze inactivation under conditions of excess calcium and high temperature (see the mechanism below). This phenomenon is displayed in the M50F mutant, which is more stable than wild type in 10mM EDTA but less stable in 10mM $CaCl_2$.

The experiment to determine stability of the calcium-free subtilisin mutants was carried out in 10mM Tris-HCl, pH 8.0, 50mM NaCl and 10mM EDTA (the association constant of EDTA for calcium is $2 \times 10^8 M^{-1}$). The proteins were dissolved in this buffer and heated to 55°C. Aliquots were removed at intervals and the activity remaining in each aliquot was determined. The kinetics of inactivation are plotted in Figure 10. Under these conditions, the half-life of the subtilisin mutants was much improved over that of subtilisin BPN'. These results indicate that subtilisins which have been mutated to eliminate calcium binding at site A have full catalytic activity and improved stability in EDTA relative to

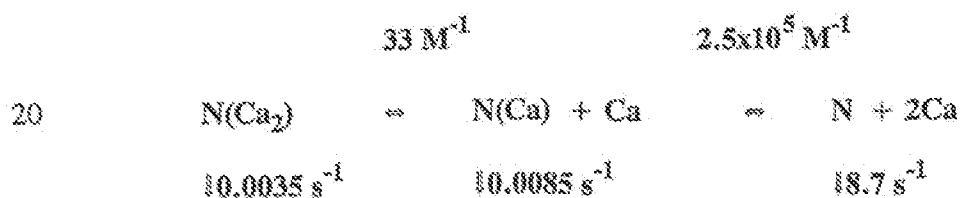
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subtilisin BPN'. A reasonable level of stability in S46 was achieved even without additional mutations to compensate for lost interactions resulting from deleting amino acids 75-83.

The inactivation of subtilisin in excess calcium is diagramed in the following mechanism:



In excess calcium (e.g. $\geq 1\text{mM}$) and moderate temperature, calcium binding and dissociation is in rapid equilibrium because calcium binding is much faster than unfolding. The rate of inactivation is determined by the fraction of each native species times its unfolding rate. Using the above mechanism, one can show that calcium dependent stabilization of subtilisin is dominated by site A rather than site B. The rate of inactivation of BPN' at 65°C as a function of calcium concentration fits the data to the following mechanism:



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U

U

U

 $k > 25 \text{ s}^{-1}$

I

The mechanism predicts that K_a 's of site A and site B are $2.5 \times 10^5 \text{ M}^{-1}$ and
 5 33 M^{-1} at 65° . The rate of inactivation of subtilisin with only site A occupied
 (NCa) is about 1000-times slower than apo-subtilisin (N) and the rate of
 inactivation with both sites occupied (NCa₂) is about 2.5-times slower than with
 only site A occupied.

The second prediction has been borne out by measuring the calcium
 10 dependent stability of a mutant which has site B but lacks site A (Strausberg et al,
Bio/Tech. 13:669-673 (1995)). The calcium-binding loop is formed from a nine
 amino acid bubble in the last turn of a 14-residue α -helix involving amino acids
 63-85 (McPhalen & James, *Biochem.* 27:6582-6598 (1988)). Deleting amino
 acids 75-83 creates an uninterrupted helix and abolishes the calcium binding
 15 potential at site A (Almog et al, *Proteins* 31:21-32 (1998); Bryan et al, *Biochem.*
31:4937-4945 (1992)). The x-ray structure has shown that except for the region
 of the deleted calcium-binding loop, the structure of the mutant and wild type
 protein are remarkably similar considering the size of the deletion. The structures
 of subtilisin with and without the deletion superimpose with an rms difference
 20 between 261 C α positions of 0.17 Å. The N-terminus of the wild-type protein lies
 beside the site A loop, furnishing one calcium coordination ligand, the side chain

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oxygen of Q2. In $\Delta 75-83$ subtilisin, the loop is gone, but the helix is uninterrupted and shows normal helical geometry over its entire length. The rate of inactivation of $\Delta 75-83$ subtilisin is only 2.4-times slower in 10mM CaCl_2 , 50mM NaCl than in 10mM EDTA, 50mM NaCl.

5 Another prediction of this last mechanism is that any mutations which stabilize only in the presence of calcium will increase the binding constant for calcium to one or both of the calcium sites. This can be either through effects on the binding sites themselves, as proposed for mutations A116E, G131D, P172D, S63D, N76D and S78D (Pantoliano et al, *Biochem.* 28:7205-7213 (1989);
10 Pantoliano et al, *Biochem.* 27:8311-8317 (1988); Rollence et al, *CRC Crit. Rev. Biotech.* 8:217-224 (1988)), or through indirect effects on conformational stability as seen for mutations S9A, I31L, S53T, L126I, , E 156S, G166S, G169A, S188P and T254A. The indirect effect on calcium binding arises because apo-subtilisin displays a loss of cooperativity in the unfolding reaction (Bryan et al,
15 *Biochem.* 31:4937-4945 (1992)). Thus many mutations which stabilize in the presence of calcium do not stabilize in the presence of EDTA, because they do not influence the rate determining step in the unfolding of apo-subtilisin. In fact, most mutations in natural subtilisins identified to date stabilize only in the presence of calcium. These mutants increase calcium binding affinity because they
20 preferentially stabilize NCa relative to N. The premise that the effects of this class of mutations indirectly increase calcium affinity by increasing general

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stability was tested using S88, a stabilize version of $\Delta 75-83$ subtilisin (Strausberg et al. 1995). The mutations S9A, I31L, E156S, G166S, G169A, N212G, S188P, K217L and T254A were introduced into the S88 version of $\Delta 75-83$ subtilisin (see Table VI).

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TABLE VI	
Mutation	Increase in stabilization over S88 subtilisin in either calcium or EDTA
S9A	1.8
I31L	1.5
L126I	2.0
E156S	1.2
G166S	2.3
G169A	5.0
N212G	1.5
M222Q	2.0
S188P	1.3
T254A	3.3
Combined in S88 subtilisin	1000

Because the unfolding of the S88 subtilisin is cooperative in EDTA, these mutations now stabilize subtilisin S88, independent of calcium concentration, to approximately the same extent that they stabilize subtilisin BPN' in 50mM Tris-HCl, pH 8.0, 50mM NaCl, 10mM CaCl_2 .

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Thus, the present inventors have provided convincing evidence that subtilisin mutants may be obtained which remain active and yet do not bind calcium. It is expected therefore that these mutants may be utilized in industrial environments that comprise chelating agents. While this has only been specifically shown with subtilisin BPN', equivalent mutations should work with other serine proteases as well, most particularly other I-S1 or I-S2 subtilisins given that these subtilisins possess substantial sequence similarity, especially in the calcium binding site.

Such strategies, for example, may involve comparing the sequence of subtilisin BPN' to other serine proteases in order to identify the amino acids which are suspected to be necessary for calcium binding and then making suitable modifications, e.g., by site-specific mutagenesis. Since many subtilisins are related to subtilisin BPN' not only through their primary sequences and enzymological properties, but also by X-ray crystallographic data, it is expected that other active subtilisin mutants which lack calcium binding may be produced by site specific mutagenesis. For example, structural evidence exists that the homologous enzyme subtilisin Carlsberg also comprises two calcium binding sites. Similarly, the X-ray structure of thermitase is known and this subtilisin has an analogous calcium A binding-site to that of subtilisin BPN'. For thermitase, the calcium binding loop is a ten residue interruption at the identical site in helix C. Accordingly, these enzymes should also be amenable to the mutations described

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herein which eliminate the calcium binding site and produce a stable, active enzyme. Moreover, as discussed supra, Siezen et al, has demonstrated that the primary calcium binding site in all subtilisins in groups I-S1 and I-S2 are formed from almost identical nine residue loops in the identical position of helix C. Thus, in view of the almost identical structures of the calcium A-sites, the methods described herein should be applicable to most if not all of the subtilisins in groups I-S1 and I-S2 set forth in Siezen et al.

Alternatively, if the amino acids which comprise the calcium binding sites are already known for a particular subtilisin, the corresponding DNA will be mutated by site specific mutagenesis to delete one or more of such amino acids, or to provide substitution, deletion or addition mutations which eliminate calcium binding.

The subject mutant subtilisins will generally be produced by recombinant methods, in particular by expression of a subtilisin DNA which has been mutated such that upon expression it results in a subtilisin protein which is enzymatically active and which does not bind calcium.

Preferably, the subtilisin DNA's will be expressed in microbial host cells, in particular *Bacillus subtilis*, because this bacteria naturally produces subtilisin, is an efficient secretor of proteins, and is able to produce the protein in an active conformation. However, the invention is not restricted to the expression of the subtilisin mutant in *Bacillus*, but rather embraces expression in any host cell

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which provides for expression of the desired subtilisin mutants. Suitable host cells for expression are well known in the art and include, e.g., bacterial host cells such as *Escherichia coli*, *Bacillus*, *Salmonella*, *Pseudomonas*; yeast cells such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluveromyces*, *Candida*,
5 *Schizosaccharomyces*; and mammalian host cells such as CHO cells. Bacterial host cells, however, are the preferred host cells for expression.

Expression of the subtilisin DNA will be provided using available vectors and regulatory sequences. The actual selection will depend in large part upon the particular host cells which are utilized for expression. For example, if the
10 subtilisin mutant DNA is expressed in *Bacillus*, a *Bacillus* promoter will generally be utilized as well as a *Bacillus* derived vector. The present inventors in particular used the pUB110-based expression vector and the native promoter from the subtilisin BPN' gene to control expression on *Bacillus subtilis*.

It is further noted that once the amino acid sequence of a particular
15 subtilisin mutant which does not bind calcium has been elucidated, it may also be possible to make the subtilisin mutant by protein synthesis, e.g., by Merrifield synthesis. However, expression of the subtilisin mutants in microbial host cells will generally be preferred since this will allow for the microbial host cell to produce the subtilisin protein in a proper conformation for enzymatic activity.
20 However, since the present inventors further teach herein a method for obtaining

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in vitro refolding of the subtilisin mutant, it should be possible to convert improperly folded subtilisin mutants into an active conformation.

In order to further illustrate the present invention and the advantages thereof, the following specific examples are given, it being understood that the same is intended only as illustrative and in nowise limitative.

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EXAMPLES

Example 1

Cloning and Expression The subtilisin gene from *Bacillus*

amyloliquefaciens (subtilisin BPN') has been cloned, sequenced, and expressed at

5 high levels from its natural promoter sequences in *Bacillus subtilis* (Wells et al.,

Nucleic Acids Res. 11:7911-7925 (1983); Vasantha et al., J. Bacteriol.

159:811819 (1984)). All mutant genes were recloned into a pUB110-based

expression plasmid and used to transform *B. subtilis*. The *B. subtilis* strain used

as the host contains a chromosomal deletion of its subtilisin gene and therefore

10 produces no background wild type (wt) activity (Fahnestock et al., Appl. Environ.

Microbial. 53:379-384 (1987)). Oligonucleotide mutagenesis was carried out as

previously described. (Zoller et al., Methods Enzymol. 100:468-500 (1983);

Bryan et al., Proc. Natl. Acad. Sci. 83:3743-3745 (1986b)). S221C was

expressed in a 1.51 New Brunswick fermentor at a level of approximately 100 mg

15 of the correctly processed mature form per liter. The addition of wild type

subtilisin to promote production of the mature form of S221C subtilisin was not

required in our bacillus host strain as was the case for prior strains (Abrahmsen

et al., Biochemistry 30:4151-4159 (1991)).

Protein Purification & Characterization. Wild type subtilisin and the

20 variant enzymes were purified and verified for homogeneity essentially as

described in Bryan et al., Proc. Natl. Acad. Sci. 83:3743-3745 (1986b);

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Pantoliano et al., Biochemistry 26:2077-2082 (1987); and Biochemistry 27:8311-8317 (1988). In some cases the C221 mutant subtilisins were re-purified on a sulfhydryl specific mercury affinity column (Affi-gel 501, Biorad). Assays of peptidase activity were performed by monitoring the hydrolysis of succinyl-
5 (L)Ala-(L)-Ala-(L)-Pro-(L)-Phe-p-nitroanilide, hereinafter sAAPFna, as described by DelMar et al., Anal Biochem, 99:316-320 (1979). The protein concentration, [P], was determined using $P^{0.1\%} = 1.17$ at 280 nm (Pantoliano et al., Biochemistry 28:7205-7213 (1989)). For variants which contain the Y217K change, the $P^{0.1\%}$ at 280 nm was calculated to be 1.15 (or 0.96 X wt), based on the loss of one Tyr
10 residue (Pantoliano et al., Biochemistry 28:7205-7213 (1989)).

N-terminal Analysis The first five amino acids of subtilisin S15 were determined by sequential Edman degradation and HPLC analysis. This revealed that 100% of the material had the amino acid sequence expected from the DNA sequence of the gene and that processing of the pro-peptide was at the same
15 position in the sequence for the mutant as for the wild type enzyme.

Example 2

Structure of the calcium A site of S12 subtilisin Calcium at site A is coordinated by five carbonyl oxygen ligands and one aspartic acid. Four of the carbonyl oxygen ligands to the calcium are provided by a loop composed of amino
20 acids 75-83 (Figure 2). The geometry of the ligands is that of a pentagonal

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bipyramid whose axis runs through the carbonyls of 75 and 79. On one side of the loop is the bidentate carboxylate (D41), while on the other side is the N-terminus of the protein and the side chain of Q2. The seven coordination distances range from 2.3 to 2.6 Å, the shortest being to the aspartyl carboxylate.

- 5 Three hydrogen bonds link the N-terminal segment to loop residues 78-82 in parallel-beta arrangement.

Preparation of apo-subtilisin S11 and S12 subtilisin contain an equal molar amount of tightly bound calcium after purification. X-ray crystallography has shown this calcium to be bound to the A site (Finzel et al., J. Cell. Biochem.
10 Suppl. 10A:272 (1986); Pantoliano et al., Biochemistry 27:8311-8317 (1988); McPhalen et al., Biochemistry 27:6582-6598 (1988)).

Complete removal of calcium from subtilisin is very slow, requiring 24 hours of dialysis against EDTA at 25°C to remove all calcium from the protein and then 48 more hours of dialysis in high salt (Brown et al., Biochemistry
15 16:3883-3896 (1977)) at 4°C to remove all EDTA from the protein. To prepare the calcium-free form of subtilisins S11 and S12, 20mg of lyophilized protein was dissolved in 5ml of 10mM EDTA, 10mM tris(hydroxymethyl)amino-methane hydrochloric acid (hereinafter Tris-HCl) at pH 7.5 and dialyzed against the same
20 buffer for 24 hours at 25°C. In order to remove EDTA, which binds to subtilisin at low ionic strength, the protein was then dialyzed twice against 2 liters of 0.9M NaCl, 10mM Tris-HCl at pH 7.5 at 4°C for a total of 24 hours and then three

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times against 2 liters of 2.5mM Tris-HCl at pH 7.5 at 4°C for a total of 24 hours. Chelex 100 was added to all buffers not containing EDTA. When versions of C221 subtilisin not containing stabilizing amino acid substitutions were used, up to 50% of the protein-precipitated during this procedure. It is essential to use pure native apoenzyme in titration experiments so that spurious heat produced by precipitation upon the addition of calcium does not interfere with the measurement of the heat of binding.

To ensure that preparations of apo-subtilisin were not contaminated with calcium or EDTA, samples were checked by titration with calcium in the presence of Quin2 prior to performing titration calorimetry.

Titration Calorimetry Measurements The calorimetric titrations were performed with a Microcal Omega titration calorimeter as described in detail by Wiseman et al., Analytical Biochemistry 179:131-137 (1989). The titration calorimeter consists of a matched reference cell containing the buffer and a solution cell (1.374 mL) containing the protein solution. Microliter aliquots of the ligand solution are added to the solution cell through a rotating stirrer syringe operated with a plunger driven by a stepping motor. After a stable baseline was achieved at a given temperature, the automated injections were initiated and the accompanying heat change per injection was determined by a thermocouple sensor between the cells. During each injection, a sharp exothermic peak appeared which returned to the baseline prior to the next injection occurring 4 minutes later. The

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area of each peak represents the amount of heat accompanying binding of the added ligand to the protein. The total heat (Q) was then fit by a nonlinear least squares minimization method (Wiseman et al., Analytical Biochemistry 179:131-137 (1989)) to the total ligand concentration, $[Ca]_{total}$, according to the equation:

$$5 \quad dQ/d[Ca]_{total} = \Delta H[1/2 + (1 - (1 + r)/2 - X_r/2)/X_r - 2X_r(1 - r) + 1 + r^2]^{1/2} \quad (1)$$

wherein $1/r = [P]_{total} \times K_d$ and $X_r = [Ca]_{total}/[P]_{total}$.

The binding of calcium to the S11 and S12 subtilisins was measured by titration calorimetry as it allows both the binding constant and the enthalpy of binding to be determined (Wiseman et al., Analytical Biochemistry 179:131-137
10 (1989); Schwarz et al., J. Biol. Chem. 266:24344-24350 (1991)).

The S11 and S12 subtilisin mutants were used in titration experiments because production of the wild type apoenzyme is impossible due to its proteolytic activity and low stability. Titrations of S11 and S12 were performed at protein concentrations $[P] = 30\mu M$ and $100\mu M$. Titration of the S11 apoenzyme with
15 calcium at 25°C is shown in Figure 4. The data points correspond to the negative heat of calcium binding associated with each titration of calcium. The titration calorimeter is sensitive to changes in K_d under conditions at which the product of $K_d \times [P]$ is between 1 and 1000 (Wiseman et al., Analytical Biochemistry 179:131-137 (1989)). Since the K_d for subtilisin is about $1 \times 10^7 M^{-1}$, these protein
20 concentrations result in values of $K_d \times [P] = 300$ and 1000. At lower protein

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concentrations the amount of heat produced per titration is difficult to measure accurately.

The results of fitting the titrations of S11 and S12 to a calculated curve are summarized in Table 2. The parameters in the table include binding parameters
5 for stoichiometric ratio (n), binding constant (K_d) and binding enthalpy (ΔH). These parameters were determined from deconvolution using nonlinear least squares minimization (Wiseman et al., Analytical Biochemistry 179:131-137 (1989)). Measurements for each experimental condition were performed in duplicate at 25°C. The protein concentrations ranged from 30 to 100 μ M while
10 the concentration of the calcium solutions were about 20 times the protein concentrations. Each binding constant and enthalpy were based on several titration runs at different concentrations. Titration runs were performed until the titration peaks were close to the baseline.

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TABLE 2: Titration Calorimetry of the Calcium A Site in Subtilisin Mutants S11 and S12.

Mutant	[P]	Parameters calculated from fit		
		n	K_a	ΔH
S11	100 μ M	0.98 ± 0.01	$7.8 \pm 0.2 \times 10^6$	-11.3 ± 0.1
S11	33 μ M	0.9 ± 0.3	$6.8 \pm 1.5 \times 10^6$	-10.9 ± 0.2
S12	100 μ M	0.99 ± 0.01	$6.4 \pm 0.2 \times 10^6$	-11.8 ± 0.5

The average values obtained are similar for S11 and S12: $\Delta H = \sim -11$ kcal/mol; $K_a = 7 \times 10^6 \text{ M}^{-1}$ and a stoichiometry of binding of 1 calcium site per molecule. The weak binding site B does not bind calcium at concentrations below the millimolar range, and therefore does not interfere with measurement of binding to the binding site A. The standard free energy of binding at 25°C is 9.3 kcal/mol. The binding of calcium is therefore primarily enthalpically driven with only a small net loss in entropy ($\Delta S_{\text{binding}} = -6.7 \text{ cal/}^\circ\text{mol}$).

Example 3

In vitro refolding of S15 subtilisin. For refolding studies subtilisin was maintained as a stock solution in 2.5 mM Tris-HCl at pH 7.5 and 50mM KCl at a concentration of approximately 100 μ M. The protein was denatured by diluting the stock solution into 5M guanidine hydrochloride (Gu-HCl) at pH 7.5 or in most cases into 25 mM H_3PO_4 or HCl at pH 1.8 - 2.0. The final concentration of protein was 0.1 to 5 μ M. S15 was completely denatured in less than 30 seconds

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by these conditions. S12 required approximately 60 minutes to become fully denatured. Acid-denatured protein was then neutralized to pH 7.5 by the addition of Tris-base (if denatured in HCl) or 5M NaOH (if denatured in H_3PO_4). Refolding was initiated by the addition of KCl, NaCl or $CaCl_2$ to the desired concentration. For example, KCl was added from a stock solution of 4M to a
5 final concentration of 0.1 to 1.5M with rapid stirring. In most cases renaturation was carried out at 25°C. The rate of renaturation was determined spectrophotometrically by uv absorption from the increase in extinction at $\lambda = 286$, from the increase in intrinsic tyrosine and tryptophan fluorescence
10 (excitation $\lambda = 282$, emission $\lambda = 347$), or by circular dichroism from the increase in negative ellipticity at $\lambda = 222$ nm.

Example 4

X-ray Crystallography. Large single crystal growth and X-ray diffraction data collection were performed essentially as previously reported (Bryan et al.,
15 Proteins: Struct. Funct. Genet. 1:326-334 (1986a); Pantoliano et al., Biochemistry 27:8311-8317 (1988); Pantoliano et al., Biochemistry 28:7205-7213 (1989)) except that it was not necessary to inactivate the S221C variants with diisopropyl fluorophosphate (DFP) in order to obtain suitable crystals. The starting model for S12 was made from the hyperstable subtilisin mutant 8350

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(Protein Data Bank entry ISO1.pdb). The S12 structure was refined and then modified to provide the starting model for S15.

Data sets with about 20,000 reflections between 8.0 Å and 1.8 Å resolution were used to refine both models using restrained least-squares techniques

- 5 (Hendrickson et al., "Computing in Crystallography" in Diamond et al., eds., Bangalore: Indian Institute of Science 13.01-13.23 (1980)). Initial difference maps for S15, phased by a version of S12 with the entire site A region omitted, clearly showed continuous density representing the uninterrupted helix, permitting an initial S15 model to be constructed and refinement begun. Each mutant was
- 10 refined from R approximately 0.30 to R approximately 0.18 in about eighty cycles, interspersed with calculations of electron density maps and manual adjustments using the graphics modeling program FRODO (Jones, J. Appl. Crystallogr. 11:268-272 (1978)).

- Except for the region of the deleted calcium-binding loop, the structures of
- 15 S12 and S15 are very similar, with a root mean square (r.m.s) deviation of 0.18 Å between 262 α-carbons. The N-terminus of S12 (as in the wild-type) lies beside the site A loop, furnishing one calcium coordination ligand, the side chain oxygen of Q2. In S15 the loop is gone, leaving residues 1-4 disordered. In S12 (as in wild type) the site A loop occurs as an interruption in the last turn of a 14-residue
- 20 alpha helix; in S15 this helix is uninterrupted and shows normal helical geometry

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over its entire length. Diffuse difference density and higher temperature factors indicate some disorder in the newly exposed residues adjacent to the deletion.

Example 5

Differential Scanning Calorimetry The stability properties of S12 and S15 were studied using DSC (differential scanning calorimetry). The $\Delta 75-83$ mutant (S15) is very similar in melting temperature to the apoenzyme of S12. The DSC profiles of apo-S12 and S15 are shown in Figure 3. The temperature of maximum heat capacity is 63.0°C for S15 and 63.5°C for apo-S12 at pH 9.63. The DSC experiments were carried out at high pH to avoid aggregation during the denaturation process. The amount of excess heat absorbed by a protein sample as the temperature increased through a transition from the folded to unfolded state at constant pressure, which provided a direct measurement of the ΔH of unfolding (Privalov et al., Methods Enzymol. 131:4-51 (1986)). ΔH_{cal} of unfolding for apo-S12 and S15 is about 140 kcal/mol. Above pH 10.0, the unfolding transition for S15 fit a two-state model reasonably well, consistent with equilibrium thermodynamics as expressed in the van't Hoff equation ($d \ln K / dT = \Delta H_{vH} / (RT^2)$) with ΔH_{vH} (the van't Hoff enthalpy or apparent enthalpy) approximately equal to ΔH_{cal} (the calorimetric or true enthalpy). At pH 9.63, however, the melting profile for both proteins was asymmetric indicating that the unfolding is not a pure two-state process.

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Example 6

Measuring kinetics of calcium dissociation. The dissociation of calcium from subtilisin is a slow process. To measure this rate the fluorescent calcium chelator Quin 2 was used. Quin 2 binds calcium with a K_d of 1.8×10^8 at pH 7.5 (Linse et al., Biochemistry 26:6723-6735 (1987)). The fluorescence of Quin 2 at 495nm increases by approximately 6-fold when bound to calcium (Bryant, Biochem. J. 226:613-616 (1985)). Subtilisin S11 or S12 as isolated contains one calcium ion per molecule. When mixed with an excess of Quin 2, the kinetics of calcium release from the protein can be followed from the increase in fluorescence at 495nm. The reaction is assumed to follow the pathway $N(\text{Ca}) \leftrightarrow N + \text{Ca} + \text{Quin } 2 \leftrightarrow \text{Quin}(\text{Ca})$. The dissociation of calcium from subtilisin is very slow relative to calcium binding by Quin 2, such that the change in fluorescence of Quin 2 is equal to the rate of calcium dissociation from subtilisin. As can be seen in Figure 5a, the initial release of calcium from S11 follows simple first order kinetics.

Temperature dependence of calcium dissociation The first order rate constant (k) for calcium dissociation was measured from 20° to 45°C. The plot of $\ln k$ vs. $1/T^\circ\text{K}$ is roughly linear. The calcium dissociation data was curve fit using transition state theory according to the Eyring equation:

$$\Delta G^\ddagger = -RT \ln K^\ddagger = -RT \ln kh/k_B T \quad (2)$$

wherein k_B is the Boltzman constant, h is Planck's constant and k is the first

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order rate constant for folding. A graph of $\ln k/k_B T$ vs. $1/T$ is shown in Figure 5b.

The data was then curve fit according to the equation (Chen et al., Biochemistry 28:691-699 (1989)):

$$\ln K^\ddagger = A + B(T_0/T) + C \ln (T_0/T) \quad (3)$$

wherein $A = [\Delta C_p^\ddagger + \Delta S^\ddagger(T_0)]/R$; $B = A - \Delta G^\ddagger(T_0)/RT_0$; $C = \Delta C_p^\ddagger/R$.

The data obtained yields the following results: $\Delta G^\ddagger = 22.7$ kcal/mol; $\Delta C_p^\ddagger = -0.2$ kcal/ $^\circ$ mol; $\Delta S^\ddagger = -10$ cal/ $^\circ$ mol; and $\Delta H^\ddagger = 19.7$ kcal/mol at a reference temperature of 25°C. A possible slight curvature of the plot would be due to a

change in heat capacity associated with formation of the transition state ($\Delta C_p^\ddagger = 0.2$ kcal/ $^\circ$ mol). ΔC_p for protein folding has been shown to be closely correlated with a change in exposure of hydrophobic groups to water (Privalov et al., Adv. Protein Chem. 39:191-234 (1988); Livingstone et al., Biochemistry 30:4237-4244 (1991)). In terms of heat capacity, the transition state therefore appears similar to the native protein. The values for ΔS^\ddagger and ΔH^\ddagger obtained from Figure 5b indicate that the transition state is enthalpically less favorable than the calcium bound form with only a small change in entropy.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as

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exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

All references cited herein are incorporated in their entirety, as if individually incorporated by reference.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: BRYAN, Philip N
ALEXANDER, Patrick
STRAUSBERG, Susan L
- (ii) TITLE OF INVENTION: CALCIUM FREE SUBTILISIN MUTANTS
- (iii) NUMBER OF SEQUENCES: 1
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 - (F) ZIP: 22313-1404
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Meuth, Donna M
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 - (C) REFERENCE/DOCKET NUMBER: 028755-021
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(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1868 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 450..1599
- (ix) FEATURE:
 - (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 772..1599
- (ix) FEATURE:
 - (A) NAME/KEY: misc_feature
 - (B) LOCATION: 450
 - (D) OTHER INFORMATION: /note= "Amino Acid Val at position 450 is fMet."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CARAAATGGA AACAAACCTT TCCACCCAGG AARCAAGCGA GTGATTGCTC CTGTGTACAT	240
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Val Arg Gly Lys Lys Val Trp Ile	
-107 -105 -100	
AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC	521
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-95 -90 -85	
ACA TCC TCT GCC CAG GCG GCA GCG AAA TCA AAC GGG GAA AAG AAA TAT	569
Thr Ser Ser Ala Gln Ala Ala Gly Lys Ser Asn Gly Glu Lys Lys Tyr	
-80 -75 -70	
ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG AGC GCC GGT AAG AAG	617
Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met Ser Ala Ala Lys Lys	
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Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys	
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Tyr Val Asp Ala Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu	
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Leu Lys Lys Asp Pro Ser Val Ala Tyr Val Glu Glu Asp His Val Ala	
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CAT GCG TAC GCG CAG TCC GTG COT TAC GCG GTA TCA CAA ATT AAA GCC	809
His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala	
1 5 10	
COT GCT CTG CAC TCT CAA GGC TAC ACT GCA TCA AAT GTT AAA GTA GCG	857
Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala	
15 20 25	
GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT COT GAT TTA AAG GTA GCA	905
Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala	
30 35 40 45	
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Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn	
50 55 60	
AAC TCT CAC GGA ACT CAC GTT GCG GCG ACA GTT GCG GGT CTT AAT AAC	1001
Asn Ser His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn	
65 70 75	
TCA ATC GGT GTA TTA GCG GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA	1049
Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val	
80 85 90	

AAA GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT AAC Lys Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn 95 100 105	1097
GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG GAC GTT ATT AAC ATG AGC Gly Ile Glu Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser 110 115 120 125	1145
CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT GAT AAA Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys 130 135 140	1193
CCC GTT GCA TCC GGC GTC GTA GTC GTT GCG GCA GCG GGT AAC GAA GGC Ala Val Ala Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly 145 150 155	1241
ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT AAA TAC CCT TCT Thr Ser Gly Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser 160 165 170	1289
GTC ATT GCA GTA GGC GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC Val Ile Ala Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe 175 180 185	1337
TCA AGC GTA GGA CCT GAG CTT GAT GTC ATG GCA CCT GCG GTA TCT ATC Ser Ser Val Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile 190 195 200 205	1385
CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT ACG TCA Gln Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser 210 215 220	1433
ATG GCA TCT CCG CAC GTT GCG GGA GCG GCT GCT TTG ATT CTT TCT AAG Met Ala Ser Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys 225 230 235	1481
CAC CCG ACG TGG ACA AAC ACT CAA GTC CCG AGC AGT TTA GAA AAC ACC His Pro Asn Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr 240 245 250	1529
ACT ACA AAA CTT GGT GAT TCT TTC TAC TAT GGA AAA GGG CTG ATC AAC Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn 255 260 265	1577
GTA CAG GCG GCA GCT CAG TAA A ACATRAAAAA CCAGCCCTTGG CCCCCCGGT Val Gln Ala Ala Ala Gln * 270 275	1629
TTTTTATAT TTTTCCTTCT CCGCATGTTT AATCCGCTCC ATAATCGACG GATGGCTCCC	1689
TCTGAAAATT TTAACGAGAA ACGCGCGGTT GACCCGCTC AGTCCCGTAA CCGCCAAGTC	1749
CTGAACCTC TCAATCGCCG CTTCCCGGTT TCCGCTCAGC TCAATGCCGT AACGCTCGGC	1809
GGCGTTTTCC TCATACCGGG AGACGGCATT CGTATCGGA TCAGRAGCAA AACTGAGCA	1868

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WHAT IS CLAIMED IS:

1. An enzymatically active subtilisin protein which has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein comprises a deletion
5 of amino acids 75-83 and at least one or more substitutions selected from the group consisting of S9A, I31L, E156S, G166S, G169A, S188P, N212G, K217L, L126I, M222Q and T254A, and optionally together with at least one or more substitutions selected from the group consisting of Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, and Q271E.
- 10 2. The subtilisin mutant of claim 1, wherein the following substitutions are made: Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, N218S, Q271E, S9A, I31L, E156S, G166S, G169A, S188P, N212G, Y217L and T254A.
3. The subtilisin mutant of claim 1, wherein the subtilisin is from a *Bacillus* strain.
- 15 4. The subtilisin mutant of claim 3, wherein the subtilisin mutant is a subtilisin 13PN' mutant, a subtilisin Carlsberg mutant, a subtilisin DY mutant, a subtilisin amylosacchariticus mutant or a subtilisin mesenticopeptidase mutant or a subtilisin Savinase mutant.

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5. The subtilisin mutant of claim 4, wherein the subtilisin mutant is a subtilisin BPN' mutant.

6. An enzymatically active subtilisin protein which has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein comprises a deletion of amino acids 75-83 and the following substitutions mutations: Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, and Q271E.

7. The subtilisin mutant of claim 6, wherein the subtilisin is from a *Bacillus* strain.

8. The subtilisin mutant of claim 7, wherein the subtilisin mutant is a subtilisin 13PN' mutant, a subtilisin Carlsberg mutant, a subtilisin DY mutant, a subtilisin amylosacchariticus mutant or a subtilisin mesenticopeptidase mutant or a subtilisin Savinase mutant.

9. The subtilisin mutant of claim 8, wherein the subtilisin mutant is a subtilisin BPN' mutant.

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10. A recombinant method which provides for the expression of an enzymatically active subtilisin protein which has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein comprises a deletion of amino acids 75-83 and at least one or more substitutions selected from the group consisting of S9A, I31L, E156S, G166S, G169A, S188P, N212G, K217L, L126I, M222Q and T254A, and optionally together with at least one or more substitutions selected from the group consisting of Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, and Q271E, said method comprising:
- 10 (a) transforming a recombinant host cell with an expression vector which contains an enzymatically active subtilisin DNA which upon expression provides for the expression of a subtilisin which does not bind calcium protein;
- (b) culturing said host cell under conditions which provide for the expression of the enzymatically active subtilisin mutant; and
- 15 (c) recovering the expressed enzymatically active subtilisin mutant from said microbial host.

11. The recombinant method of claim 10, wherein the subtilisin mutant lacks the calcium A binding site.

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12. The recombinant method of claim 10, wherein the subtilisin mutant is a subtilisin BPN' mutant.

13. The recombinant method of claim 12, wherein the subtilisin BPN' DNA comprises one or more additional mutations which provide for enhanced thermal stability or which provide for restoration of cooperativity of folding of the subtilisin protein.

14. A recombinant method which provides for the expression of an enzymatically active subtilisin protein which has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein comprises a deletion of amino acids 75-83 and the following substitutions mutations: Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, and Q271E, said method comprising:

- (a) transforming a recombinant host cell with an expression vector which contains an enzymatically active subtilisin DNA which upon expression provides for the expression of a subtilisin which does not bind calcium protein;
- (b) culturing said host cell under conditions which provide for the expression of the enzymatically active subtilisin mutant; and
- (c) recovering the expressed enzymatically active subtilisin mutant from said microbial host.

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15. The recombinant method of claim 14, wherein the subtilisin mutant lacks the calcium A binding site.

16. The recombinant method of claim 14, wherein the subtilisin mutant is a subtilisin BPN' mutant.

5 17. The recombinant method of claim 16, wherein the subtilisin BPN' DNA comprises one or more additional mutations which provide for enhanced thermal stability or which provide for restoration of cooperativity of folding of the subtilisin protein.

18. A recombinant DNA which encodes for a subtilisin protein which
10 has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein comprises a deletion of amino acids 75-83 and at least one or more substitutions selected from the group consisting of S9A, I31L, E156S, G166S, G169A, S188P, N212G, K217L, L126I, M222Q and T254A, and optionally together with at least
15 one or more substitutions selected from the group consisting of Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, and Q271E, and which mutated subtilisin protein retains enzymatic activity and stability.

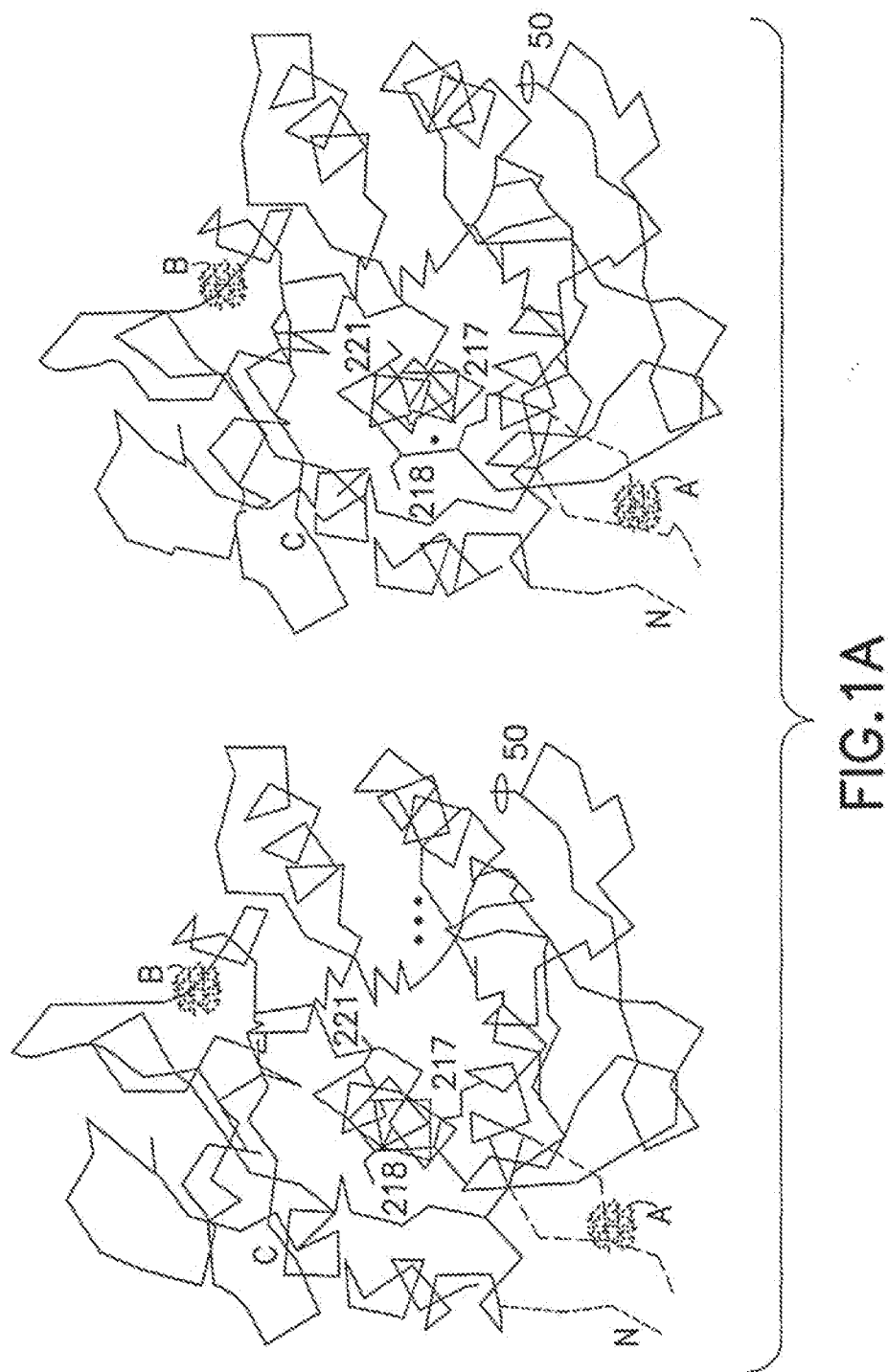
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19. The recombinant DNA of claim 18, wherein the subtilisin DNA is a subtilisin BPN' coding sequence.

20. A recombinant DNA which encodes for a subtilisin protein which has been mutated to eliminate the ability of said subtilisin protein to bind calcium at a high affinity calcium binding site, wherein the mutated subtilisin protein
5 comprises a deletion of amino acids 75-83 and the following substitutions mutations: Q2K, S3C, P5S, K43N, M50F, A73L, Q206C, Y217K, N218S, and Q271E, and which mutated subtilisin protein retains enzymatic activity and stability.

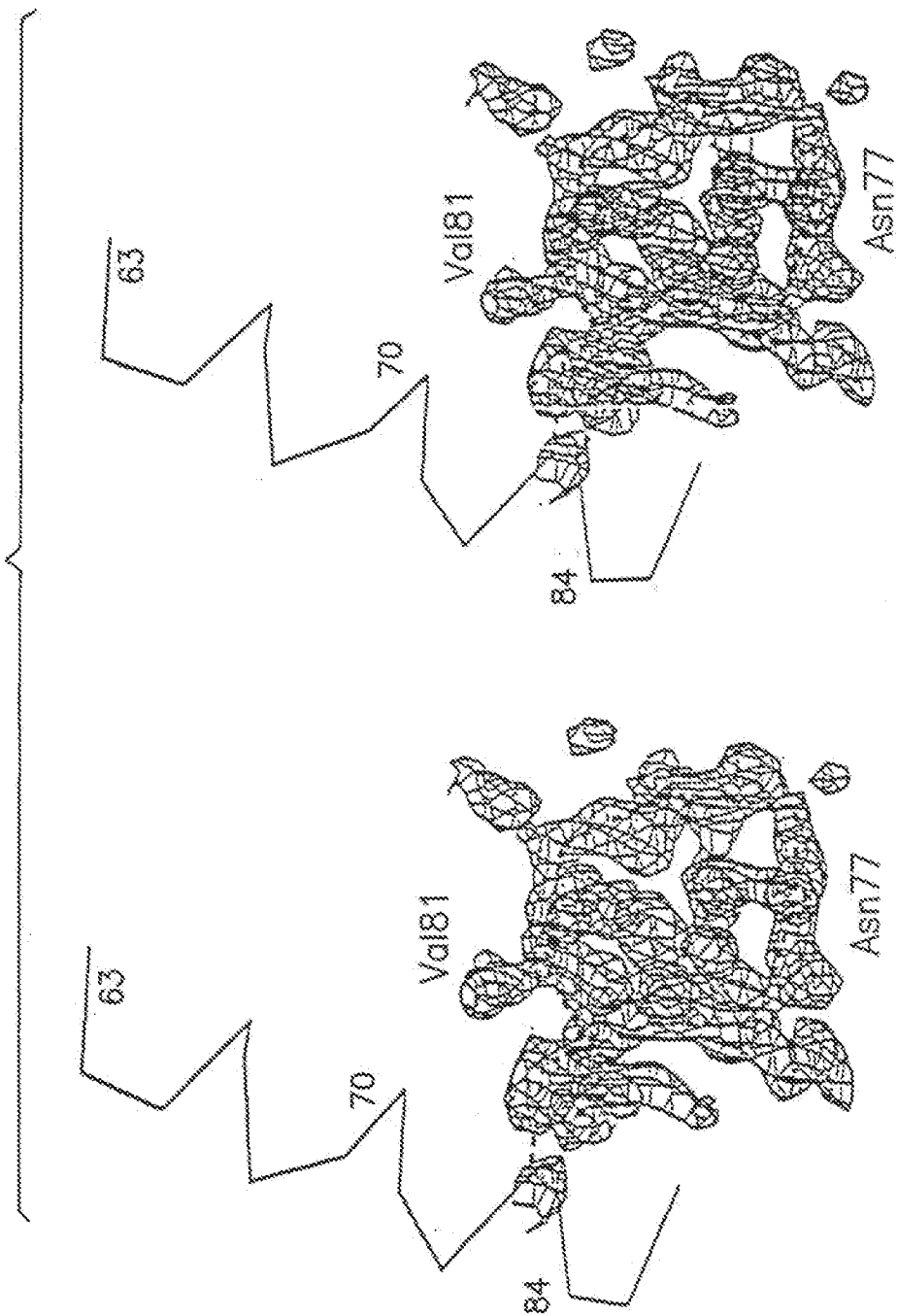
10 21. The recombinant DNA of claim 20, wherein the subtilisin DNA is a subtilisin BPN' coding sequence.

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FIG.1B



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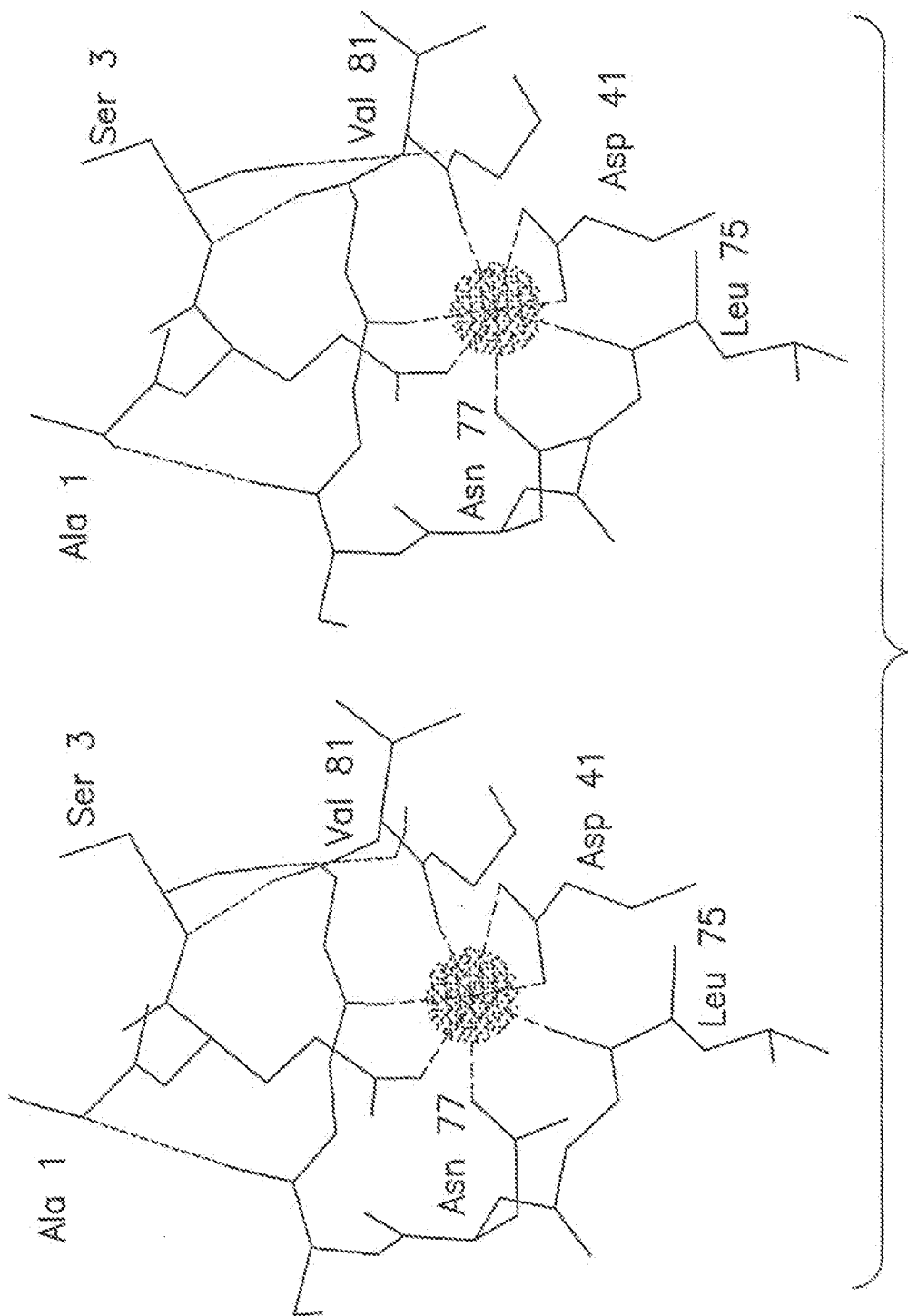


FIG.2

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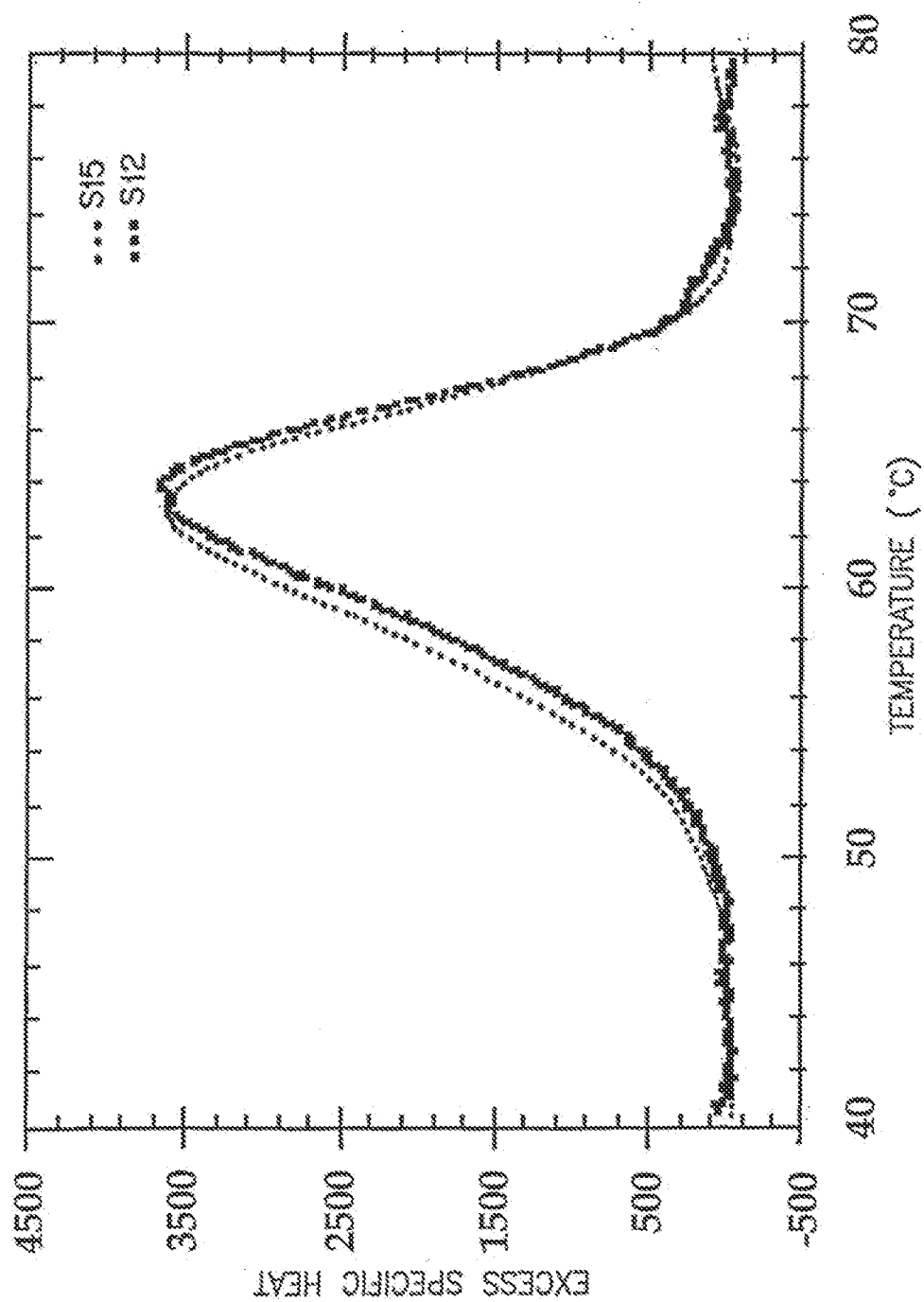


FIG.3

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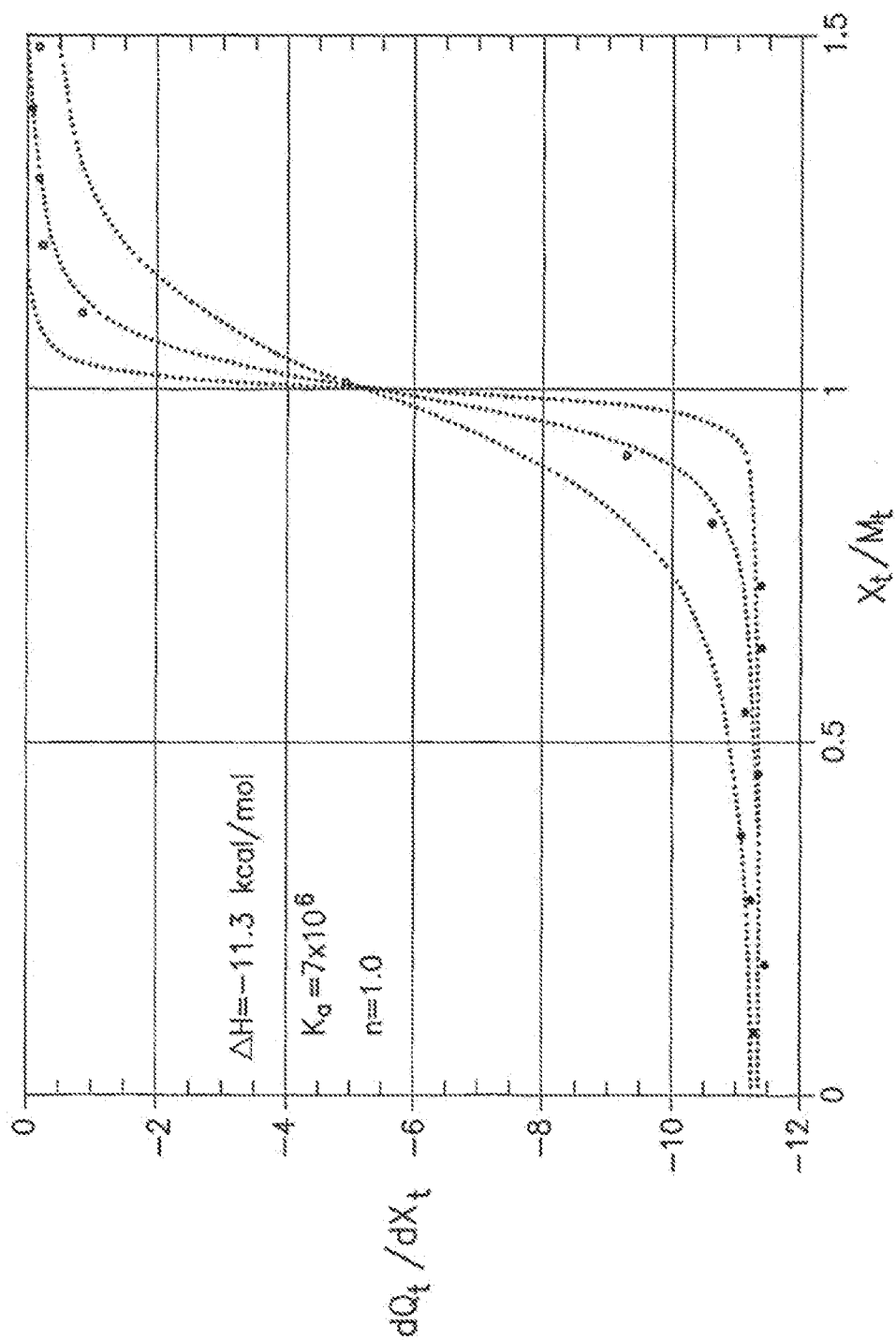
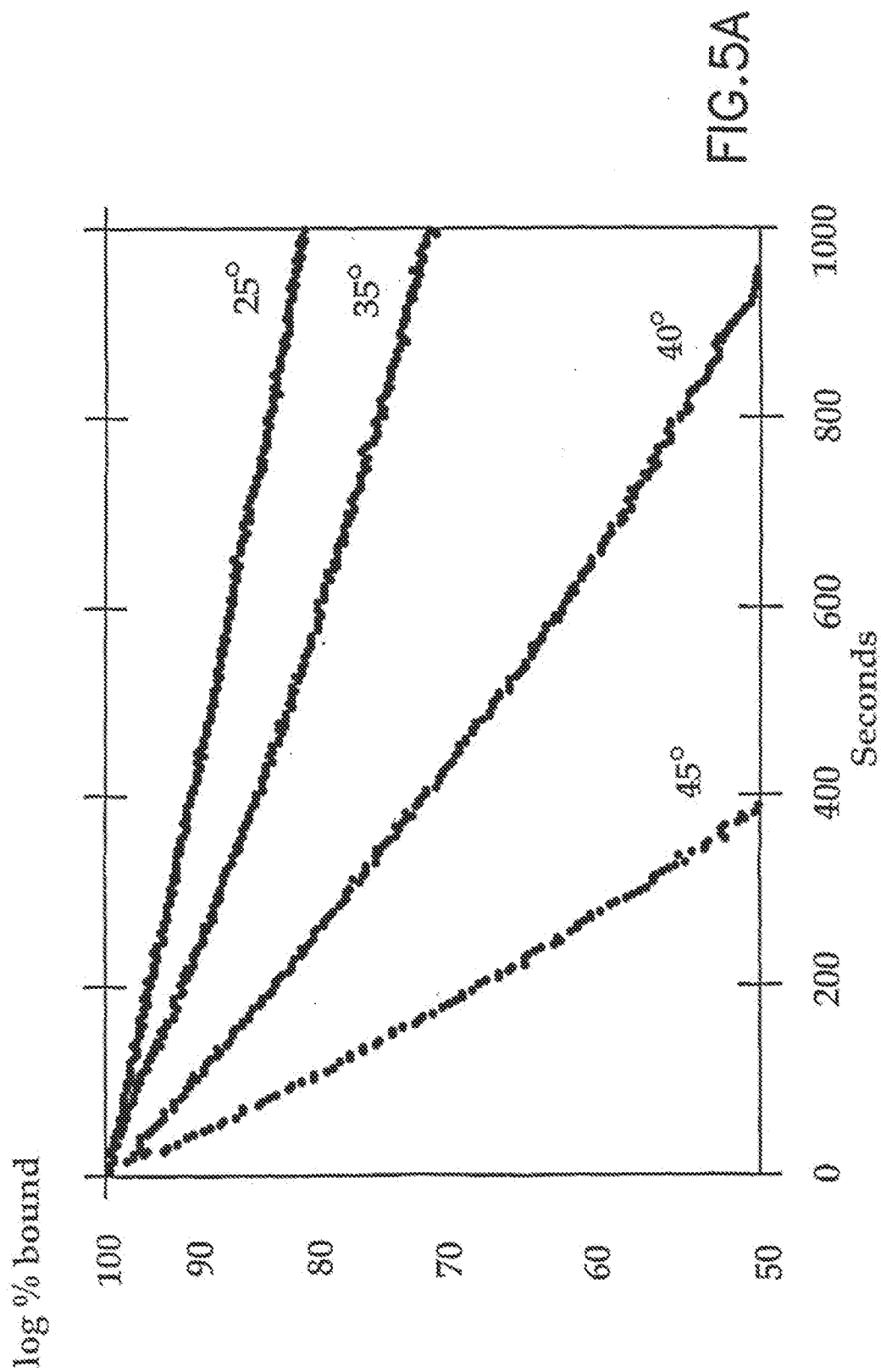


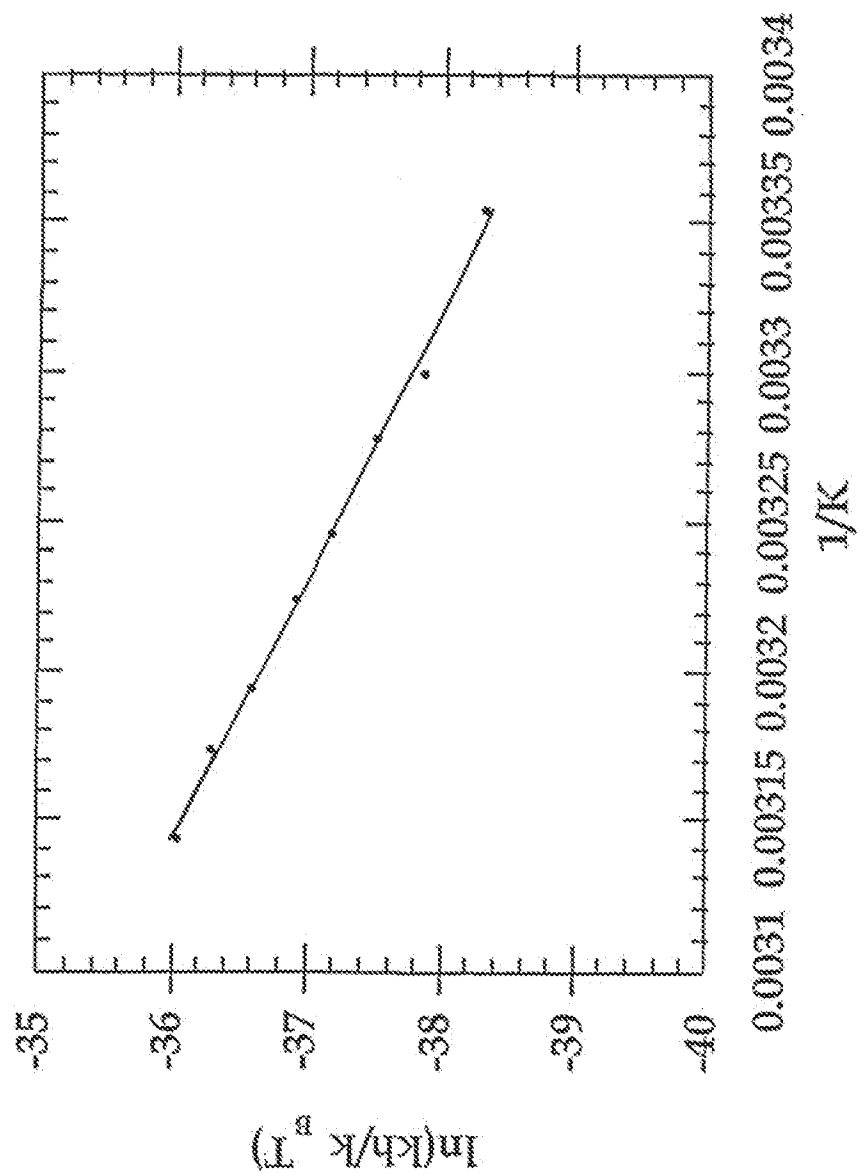
FIG. 4

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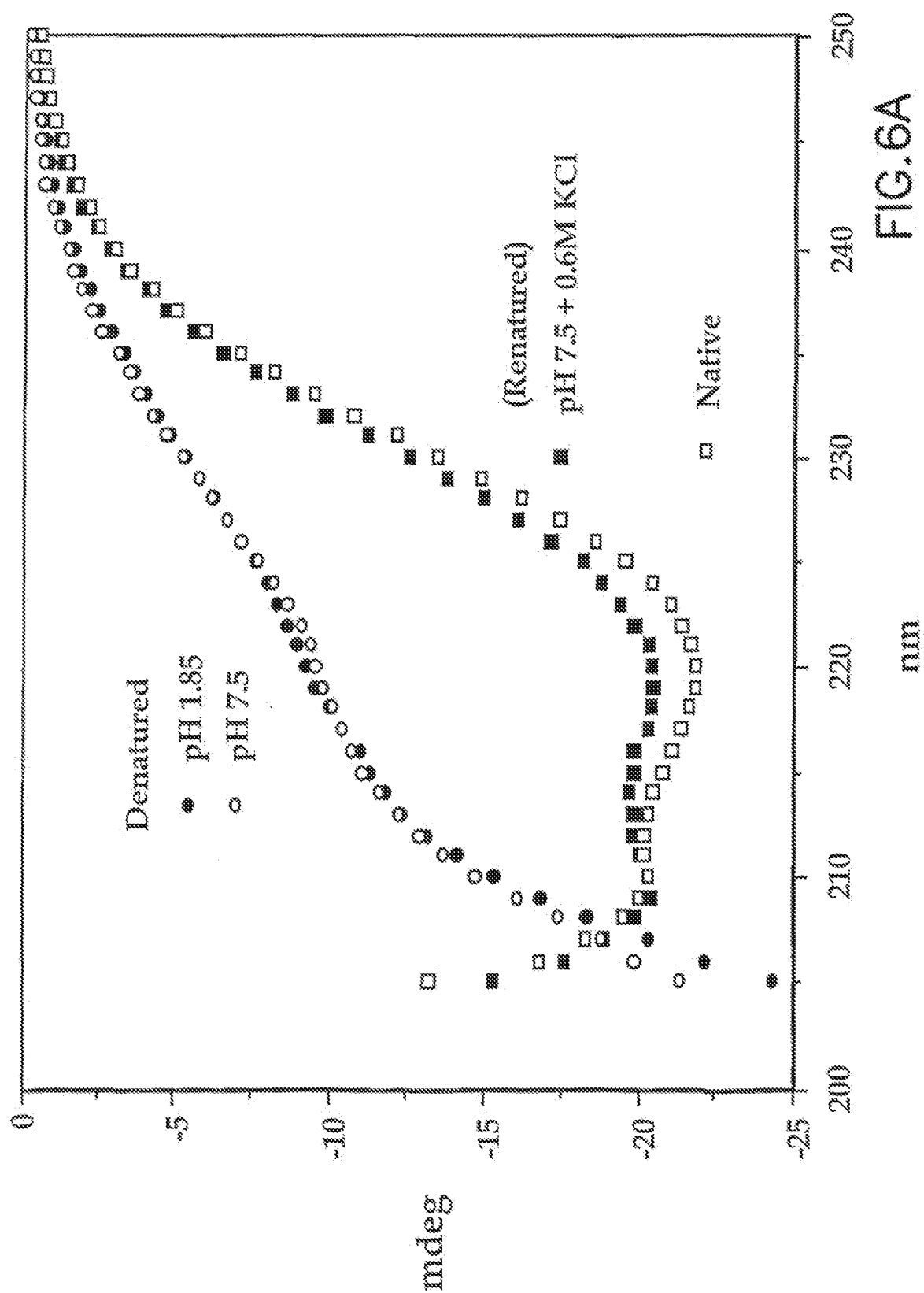


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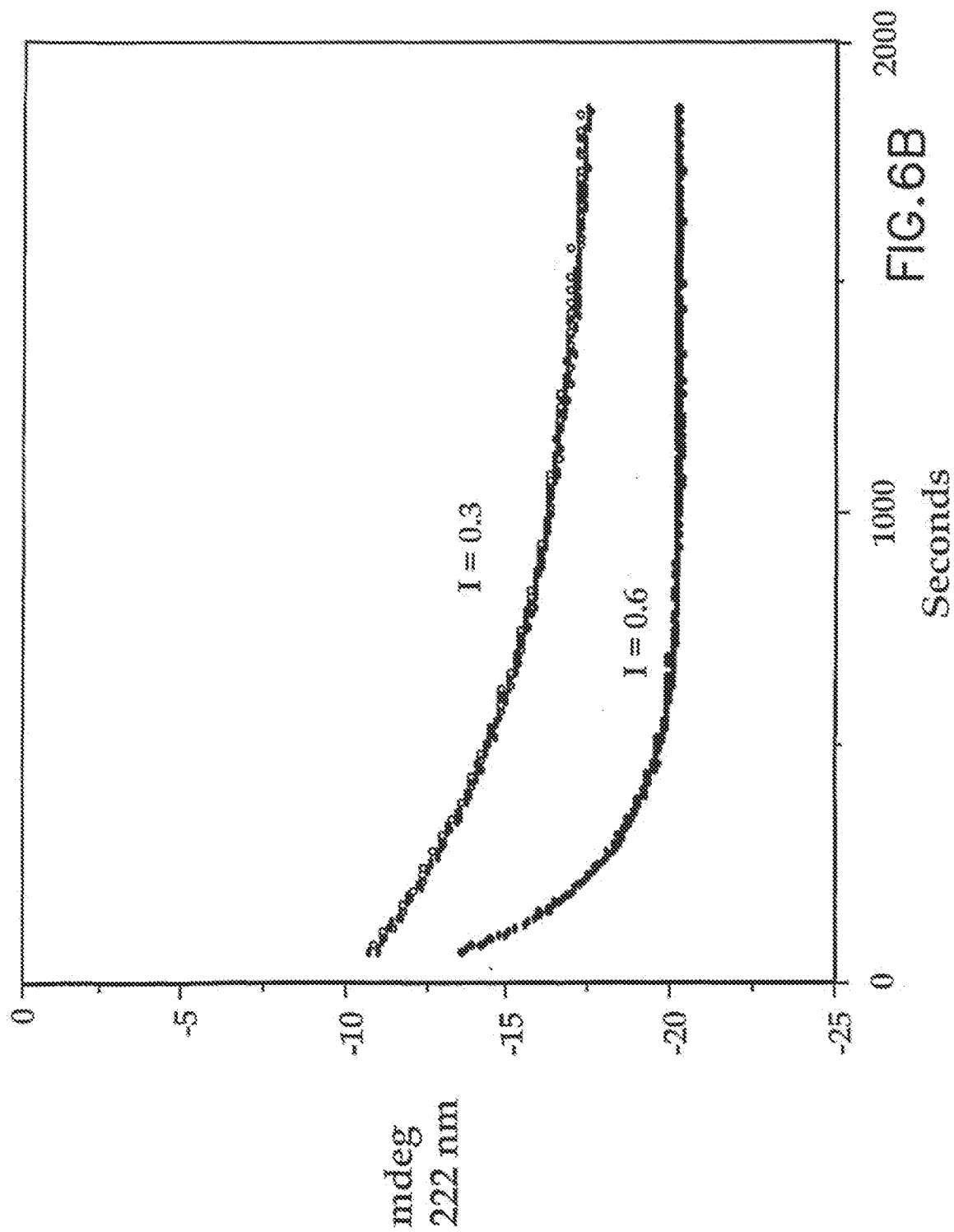
FIG. 5B



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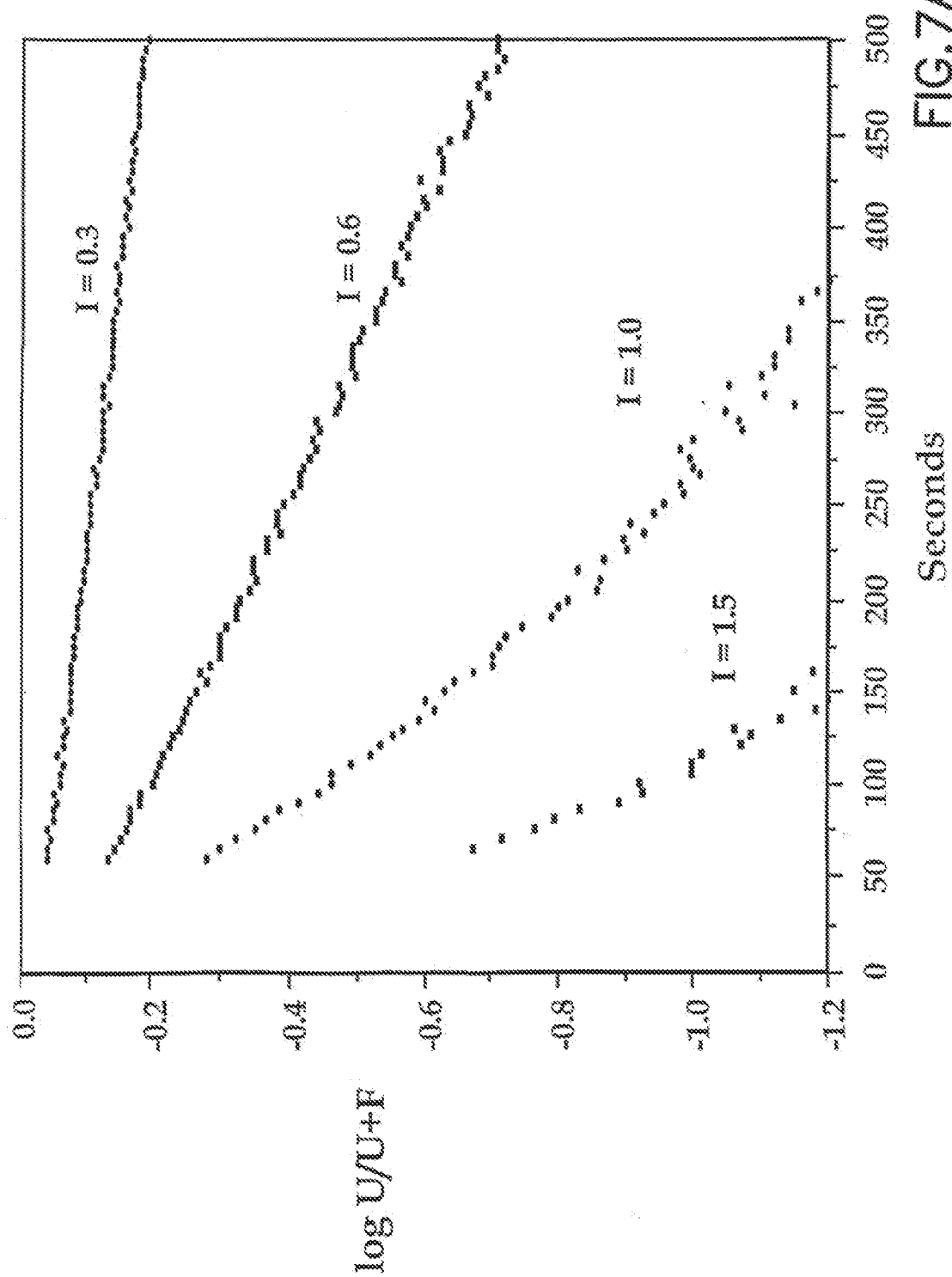
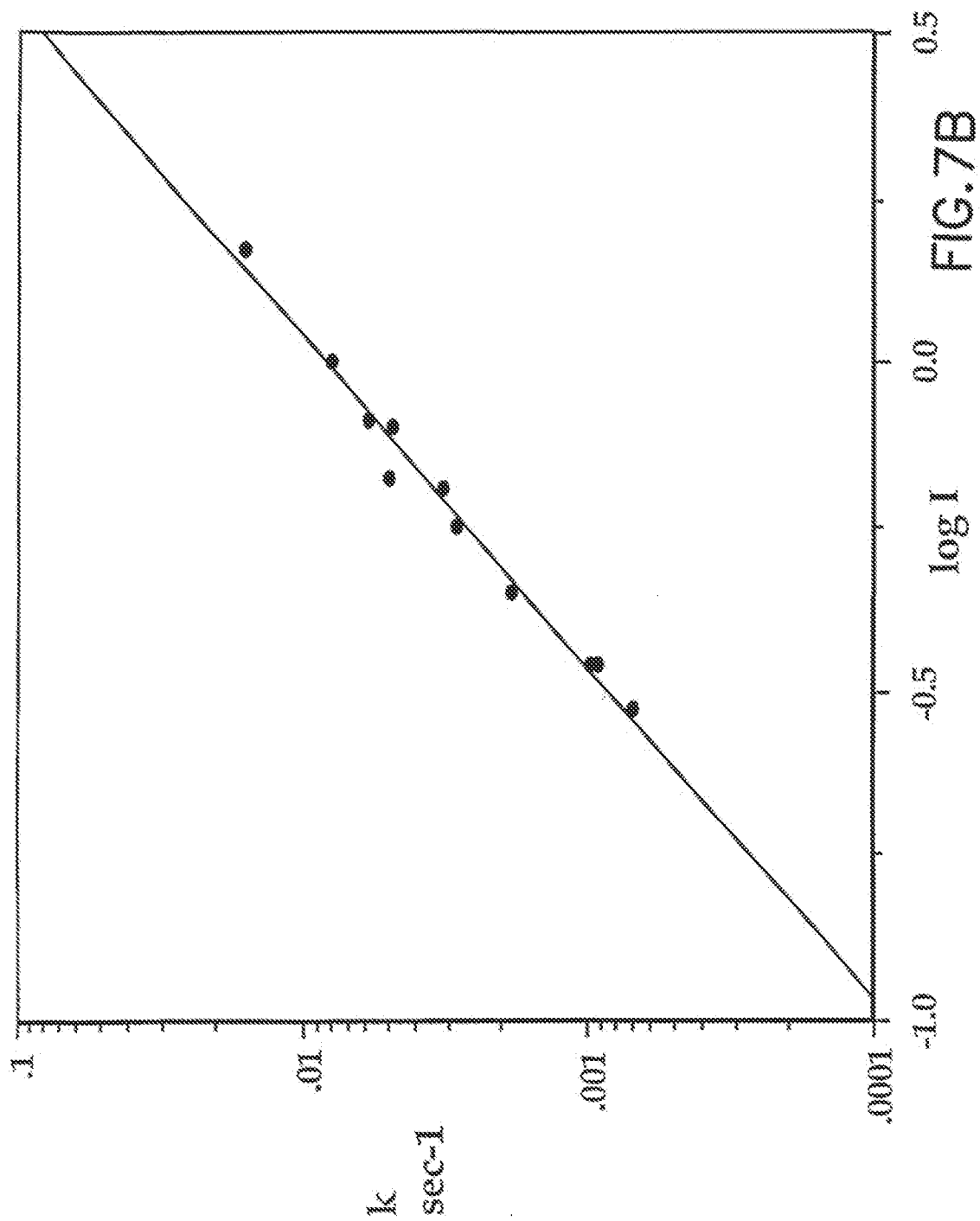


FIG. 7A

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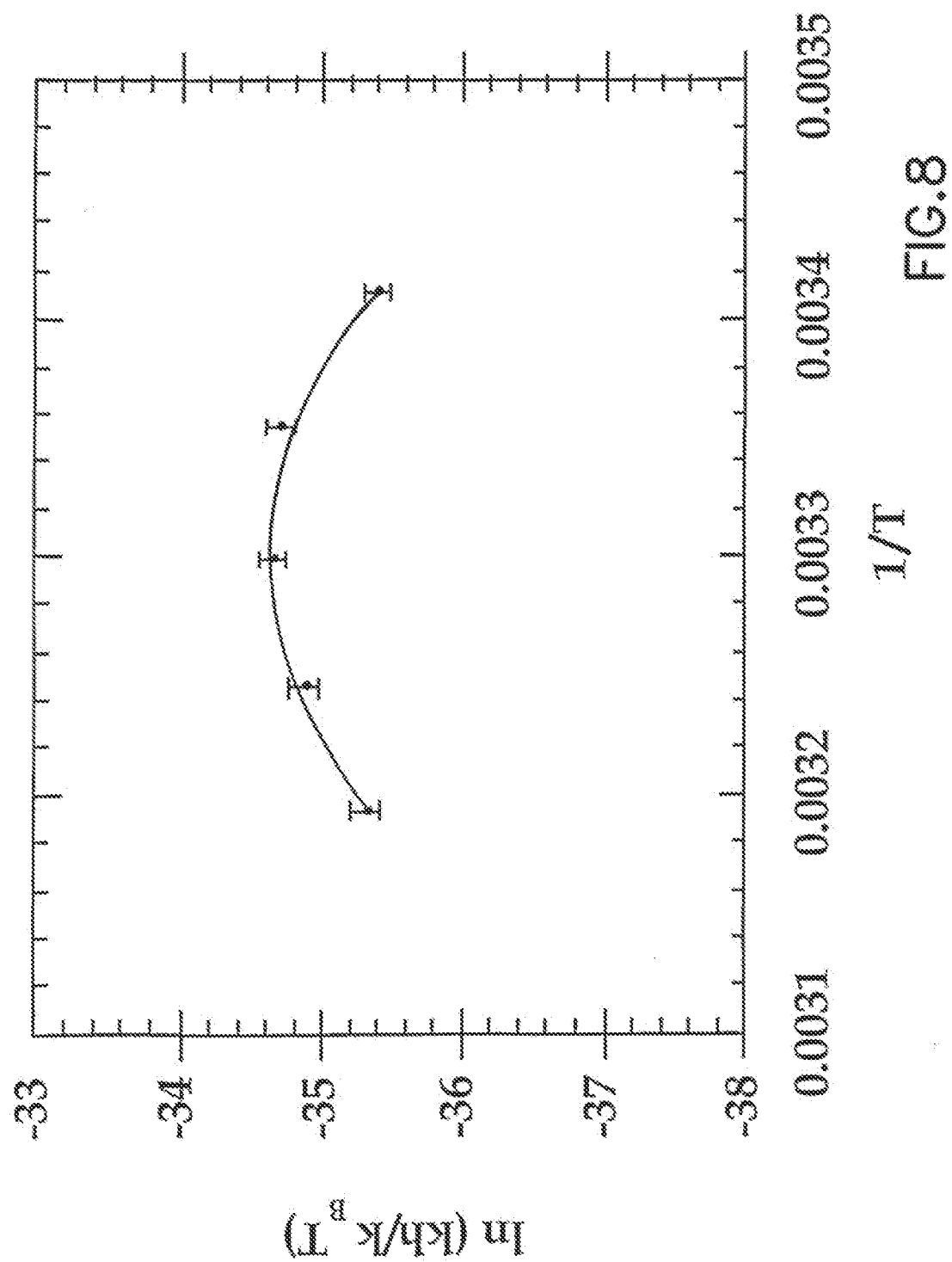
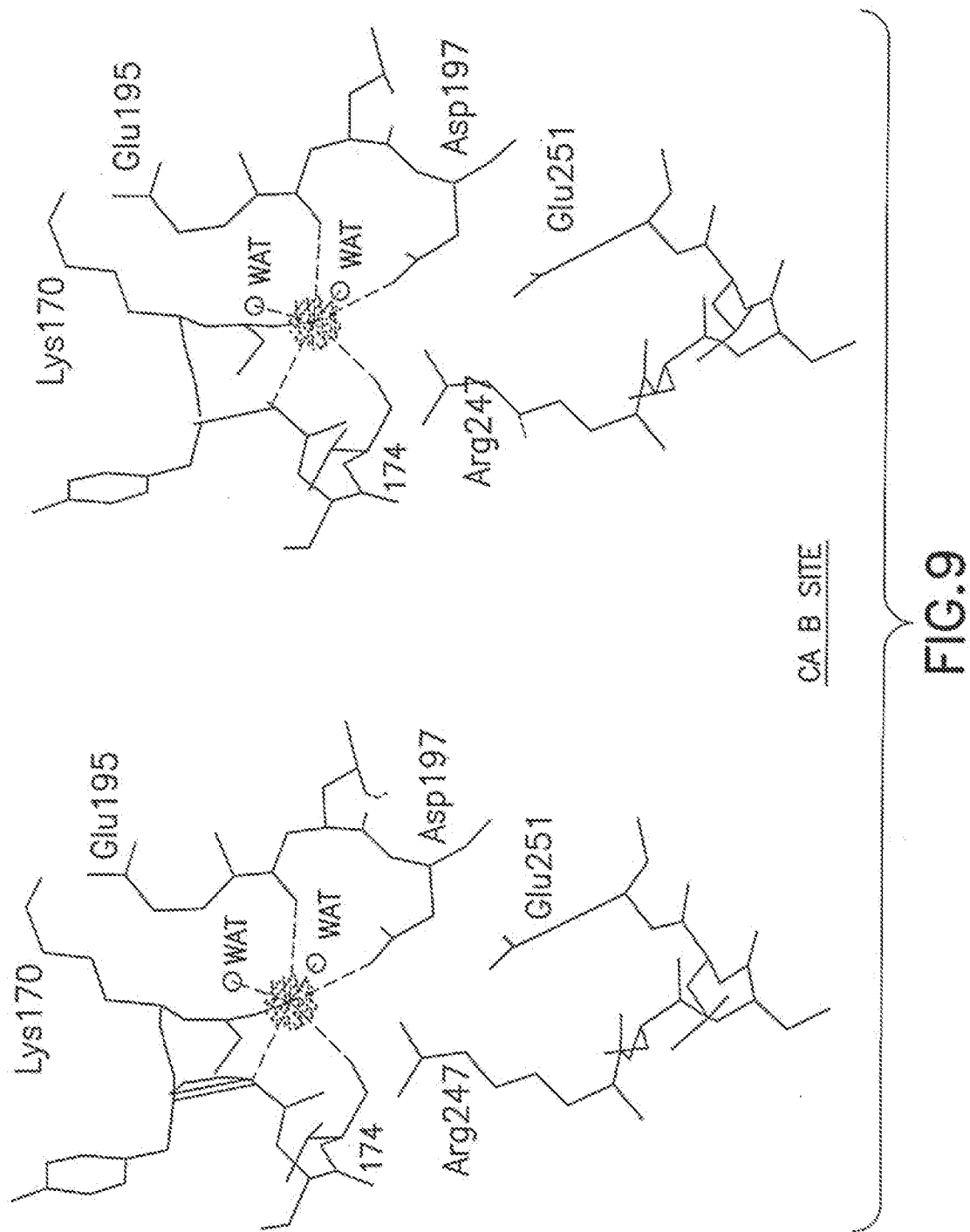


FIG.8

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/28581

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 9/54, 15/11, 15/57, 15/74

US CL : 435/221, 69.1, 252.31, 320.1, 471; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/221, 69.1, 252.31, 320.1, 471; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST Search with terms subtilisin, calcium(ad)free, and stabiliz55

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,567,601 A (BRYAN et al) 22 October 1996, see cols. 6-26, Tables I-V, especially the S68 and S86 mutants, and Figs. 1A, 1B, 2-4, 5A, 5B, 6A, 6B, 7A, 7B, 8 and 9.	1 and 3-21
Y	US 4,980,288 A (BRYAN et al) 25 December 1990, see cols. 2-16, non-numbered tables at col. 8, especially Tables I and II at cols. 15 and 16, and Figures 4-8.	1 and 3-21
Y	US 5,116,741 A (BRYAN et al) 26 May 1992, see cols. 5-21, Tables I and II, especially the GX8321, GX8324, GX8306 and GX8315 mutants, and Figs. 1-3.	1 and 3-21
Y	US 5,801,038 A (BOTT et al) 01 September 1998, see cols. 4-6, 42-44, 51-52, 57-58, 67-82, especially cols. 71-80, claims 1-15, and Figs. 5A-1, 5A-2, 5B-1, 5B-2, 12, 23A, 23B, 24, 30A-C, 32 and 33.	1 and 3-21
Y	US 5,316,935 A (ARNOLD et al) 31 May 1994, see cols. 3-10, 12-24, Tables I-V, especially the 7M, PC1 and PC3 mutants, and Figs. 2-4.	1 and 3-21
A	US 5,707,848 A (BRYAN et al) 13 January 1998, see cols. 5-20 and Tables I and II.	1 and 3-21
A	US 5,470,733 A (BRYAN et al) 28 November 1995, see cols. 5-20 and Tables I and II.	1 and 3-21

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	* T	* X	* Y	* R
"A" document defining the general state of the art which is not considered to be of particular relevance	most document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	document member of the same patent family
"E" earlier application or patent published on or after the international filing date				
"I" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)				
"O" document referring to an oral disclosure, use, exhibition or other means				
"P" document published prior to the international filing date but later than the priority date claimed				

Date of the actual completion of the international search

25 October 2001 (25.10.2001)

Name and mailing address of the ISA/US

Consultation of Patents and Trademarks

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Facsimile No. (703)305-3230

Date of mailing of the international search report.

20 NOV 2001

Authorized officer

Ponnathuram Achutanurthy

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